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STUDIES ON THE LUNG AFTER LIGATION OF THE PULMONARY ARTERY

II. ANATOMICAL CHANGES*

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The study of the collateral circulation of the lung is assuming new significance as methods are being developed for its clinical evaluation, and as its implications concerning the work of the heart are becoming understood. A great and rapid rise in the amount of blood circulating through the bronchial arteries in the dog after ligation of the pulmonary artery was demonstrated in bronchospirometric studies reported elsewhere.[1] The anatomical changes in the lungs of such animals are the subject of the present report.

Schlaepfer,[2,3] in similar experiments, observed that the bronchial arteries were greatly enlarged, but he did not follow them far into their intrapulmonary ramifications. Observations dealing with the effect of ligation of the pulmonary artery to a single lobe were made by Mathes, Holman, and Reichert.[4] By means of a roentgenographic technic involving the injection of bismuth oxychloride in acacia (Hill's mass), and in a specimen cleared by the Spalteholz method, they demonstrated the great increase of the collateral circulation and the retrograde filling of the pulmonary artery with this material. The early work has been reviewed by Berry, Brailsford, and de Burgh Daly.[5]

For an accurate study of the relations of the vessels it is advantageous to see them in the round and to be able to manipulate them. The preparation of durable casts of the bronchovascular structures serves this purpose and was employed in the present observations.

METHODS

The details of the method of producing bronchovascular casts in vinylite plastic have been presented elsewhere.[6] Briefly, the procedure

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consists of the injection of black vinylite into the aorta while the lungs remain *in situ*. The lungs are then inflated in a vacuum jar and the pulmonary arteries and veins are injected with red and green plastic respectively by means of cannulas brought out through the cover of the jar. The bronchi are finally filled with white plastic and the specimen is allowed to harden. Blocks can be removed at this stage for histologic study. The tissue is then corroded away in concentrated hydrochloric acid, leaving a multicolored cast that exhibits the bronchi and vascular structures of the lung in three dimensions. By varying the procedure, injections of vessels of any desired size can be achieved. With experimental material, the entire thorax and its contents can be suspended in the vacuum jar, after injection of the aorta *in situ* through an intercostal space. This has the advantage of confining the lungs within their natural cavities during inflation, and maintaining intact the internal mammary vessels and their branches. After digestion, a cast of the main vasculature of the entire thoracic cage remains in its proper relations with the bronchovascular structures.

Gross Observations

Origin and Course of the Bronchial Arteries in the Dog

Bruner and Schmidt,[7] in their dissections of 75 dogs, found that the right bronchial artery came from the 5th or 6th intercostal artery (1st or 2nd aortic intercostal) in 60 per cent of the cases. In the remainder, the blood supply was stated to come from the internal mammary artery or other vessels.

In our own less extensive observations, of 11 bronchovascular casts, the main supply of the bronchial trees of both sides was invariably found to be derived from the right intercostal vessels (Table I). In no case

Table I

Source of Principal Bronchial Arteries in Eleven Dogs

Source		Number
Right	Left	
A_2	A_2	3
A_3	A_3	3
A_2	A_3	2
A_1	A_2	1
A_1	A_1 and A_2	1
A_2	A_1 and A_2	1
Total		11

"A" refers to aortic intercostal.

did a left intercostal artery, or the aorta itself, give rise to a major bronchial branch, although small supplementary branches were observed in three instances. Major branches to the left side from these sources were observed in the dog by Berry, Brailsford, and de Burgh Daly.[5] The most common source was the 2nd or 3rd aortic intercostal artery supplying the 6th or 7th intercostal space. The same intercostal vessels usually supplied the esophagus, sometimes by a trunk in common with the bronchial artery. The existence of an accessory supply from many other arteries is implied by the huge collaterals that develop from them after ligation of the pulmonary artery.

The bronchial arteries in the normal dog branch and anastomose profusely within the walls of the bronchi, where their major trunks tend to pursue a spiral course. In contrast with the pulmonary arteries, these vessels are anything but end arteries. Since some of the branches of the bronchial arteries soon pass into the superficial layers of the mucosa where they anastomose with other arterioles, nothing short of complete transection of the bronchus will interrupt the flow of blood. Thus it is most unlikely that the arterial supply to the nerves of the lung can be easily interrupted in the intact animal, as it can, with damage to reflex function, in the heart-lung preparation.[5,8]

According to Miller,[9,10] a common capillary bed with the pulmonary artery is normally found only in the walls of the respiratory bronchioles. His experiments, and those of Ghoreyeb and Karsner,[11] suggest that the capillaries of the alveoli are perfused from the bronchial arteries if the pressure in the pulmonary arteries falls sufficiently low.

The Bronchial Circulation after Ligation of the Pulmonary Artery

A well developed collateral circulation was already apparent in the earliest available successful bronchovascular cast, from an animal that died 9 weeks after ligation of the left pulmonary artery. Mathes, Holman, and Reichert[4] found by their roentgenographic technic that the bronchial vessels were already moderately enlarged 7 days, and markedly enlarged 16 days, after ligation of a lobar pulmonary artery. Schlaepfer,[2] however, remarked that the dilatation was first noticeable in gross dissections after 66 days.

In the casts derived from animals dying 2¼ to 23½ months after ligation, a rough correlation could be made between the extent of the collateral circulation and the length of survival after operation. Dog 33, however, which survived 12½ months, had the most extensive collateral circulation, surpassing that of dog 9, the longest survivor.

Some conception of the appearance of the expanded bronchial circulation can be drawn from a study of the cast of dog 15, which died

at the end of the third month. In contrast with the normal (Fig. 1), in which the vinylite injection mass penetrated only to branches of the bronchial artery of the 4th to the 6th order, the black plastic here extended much farther out in the bronchial tree, especially on the left side (Figs. 2 and 3). In this instance, the bronchial arteries to both sides arose from a common trunk derived from the second right aortic intercostal artery. This parent trunk, after giving origin to the left superior bronchial artery, divided into a left inferior bronchial artery and the single right bronchial artery. The common derivation of both left and right bronchial arteries may account for the dilatation of the latter. Each of the two left bronchial arteries measured 2 mm.* at the source. The superior artery looped over the left stem bronchus, and the inferior passed along the lower, medial aspect of the lower lobe bronchus. These two vessels were joined by an almost angiomatous rete of large arterial channels which embraced each of the major bronchi of the left side. Added to this plexus were a large communicating branch from the right bronchial artery and many fine recurrent branches from an esophageal artery that was itself derived mainly from the left inferior bronchial artery, and indirectly from another esophageal artery stemming from the third right aortic vessel.

As the lungs lay *in situ,* additional sizeable collaterals from the phrenic, pericardiophrenic, and distal branches of the esophageal arteries were seen to enter the lung through the pulmonary ligament (Fig. 4). Here some of them ramified in stellate fashion within the superficial layers of the pleura where they are said not to exist normally in the dog.[10] In the dissection of the lung from the thorax, although in general the pleural cavity was free, dense fibrous adhesions were noted between portions of the upper lobe and the parietal pleura in the bed of the operative incision. Within these, numerous small vessels derived from the enlarged distal portions of the 4th and 5th intercostal arteries were seen to enter the lung. These were constantly found in all of the animals and are illustrated from another instance (Fig. 5).

As the vessels were traced distally about the bronchi of the left lung, their enormously increased size accentuated their spiral course and the plexiform anastomosing arrangement of their branches. There was little diminution in the caliber of the branches in comparison with the parent stem, and sometimes these vessels were so varicose that there might actually be an increase in the size of a distal segment. The arrangement of these vessels was identical with that of the bronchial collaterals seen in cases of bronchiectasis[12] and congenital heart disease[13] in man.

* These dimensions refer to the casts and therefore to internal diameters.

In dog 15, 3 months after operation, there was only suggestive evidence in a few places of retrograde injection of some of the pulmonary arterioles. Retrograde injection of the pulmonary arteries from the bronchials was observed 10½ months after ligation of the left stem pulmonary artery in dog 11. The most striking example of this process was in another animal, dog 33, sacrificed at 12½ months. Inspection of the upper lobe bronchus of this dog revealed a pulmonary artery regularly distributing one branch to each branch of the bronchus (Fig. 6). The tortuous bronchial arteries, of which there were several intercommunicating branches to each bronchus, were more intimately related to the lumen than the pulmonary vessels. The main bronchial arterial trunk accompanying the 3rd order branches of the lobar bronchus was only slightly smaller than the pulmonary artery at the same level. The points of communication between the bronchial and pulmonary vessels were beyond the 6th order branches of the latter. These communicating vessels were usually multiple and formed a basketwork about the bronchioles. Retrograde injection with the vinylite indicated that the vessels had reached a diameter in excess of 50 μ. Some of these vessels had a diameter as large as 200 μ. Their appearance in another animal (dog 11) was identical with that described here for dog 33 (Fig. 7).

It is surprising to find anastomoses even at the 6th order branches of the lobar bronchus, when one considers that normally the common capillary beds of the pulmonary and bronchial arteries are found only in the walls of the respiratory bronchioles. Apparently, certain of the capillaries in this bed become so widely dilated as to reach a macroscopic size. Thus the tissues at their origin are pushed aside and the bridge between the vessels appears to occupy a more central position. Mathes, Holman, and Reichert[4] stated, on the basis of a preparation cleared by the Spalteholz method, that such anastomoses were single and along the 3rd order bronchi. The vinylite casts, which have the advantage of manipulability, and which show the bronchovascular tree as a whole, have in no instance revealed them that close to the hilum. No precapillary communications between the bronchial arteries and the pulmonary veins were demonstrated.

As the vessels were traced from the periphery toward the hilum, there could be seen in the interlobar fissure and posteriorly, the well filled main pulmonary artery which terminated in a rounded protuberance at the confluence of the upper and lower lobe arteries. Here the retrogradely injected pulmonary artery was enshrouded in a close-meshed rete of collateral vessels which coursed subpleurally in the depths of the fissure and through the posterior mediastinum (Fig. 8). These bronchial arter-

ies merely lay adjacent to, but did not communicate with, the main pulmonary arteries near the heart, but only far at the periphery where the anastomoses have been described. The plexus of collateral arteries was derived from a left bronchial trunk 3.5 mm. in internal diameter which sprang, in common with a right bronchial artery, from the 3rd right aortic intercostal (Fig. 9).

Even where no retrograde injection with the vinylite from the bronchial arteries had occurred, complete patency of the main pulmonary artery distal to the point of ligature was demonstrated by injection in all four instances where it was attempted (Fig. 10). Schlaepfer[2,3] has previously observed that the pulmonary artery distal to the ligature does not become thrombosed, although he described it as "contracted." In the present experiments, the distal pulmonary arteries of the left side appeared only slightly, if at all, smaller than those of the same order on the right side, when injected with the same material at the same pressure. Obviously, the failure of retrograde injection of the pulmonary arteries from the bronchial arteries in these specimens does not indicate that the systems do not communicate by means of channels of lesser diameter than 50 μ.

Histologic Observations

Microscopic study of the bronchi and lungs confirms what is seen grossly in the casts. The bronchial arteries not only become enormously increased in size, but new, or previously minor, channels become greatly thickened (compare Figs. 11 and 12). They are prominent not only about the bronchi but also in the septa and in some portions of the pleura.

It is difficult to compare the cross-sectional areas of the pulmonary arteries with those of the bronchial arteries about the largest bronchi, since the latter vessels here are so tortuous that many are met obliquely, or met more than once, in section. About the terminal bronchi, however, the bronchial arteries are arranged in less tightly coiled spirals and a more significant comparison is possible. Here the bronchial arteries are usually multiple. Their total area in cross section generally exceeds that of the pulmonary artery that accompanies the terminal bronchus (Fig. 13). The latter vessel at this point, however, is usually larger in cross-sectional area than the largest of the comitant bronchial arteries.

The walls of the enlarged collateral vessels consist chiefly of a thick layer of large smooth-muscle fibers. There is muscular hypertrophy as well as hyperplasia (compare Figs. 14 and 15). Foci of subendo-

thelial fibrosis do not significantly encroach upon the lumen. The internal elastic lamella is irregularly thickened and fragmented, and even in the largest vessels only small segments of elastica remain. In the relatively normal bronchial arteries which accompany the main bronchi of the right side there is a complete internal elastic membrane (Fig. 14). The main pulmonary arteries appear to have well preserved fibro-elastic walls (Fig. 16).

An increase of elastic tissue in the walls of the alveoli and of the collagenous tissue about the bronchovascular rays was noted by Schlaepfer[2,3] and was thought by him to account for the shrinkage of the lung to two-thirds of its original size. In the present material the pleura and adventitial tissues of the bronchi likewise appear slightly thickened. The alveoli, however, are slightly thickened, if at all, and are indistinguishable in size from those of the relatively normal right lung in the same animal. Their capillaries do not appear to be especially engorged. No anatomical impediment to their function appears to exist.

Discussion

After a study of such specimens as are demonstrated in Figures 8 and 9, the large collateral flows (often in excess of 1 liter/M^2/min.) observed in the quantitative physiologic studies[1] become less surprising. In comparison with the normal pulmonary arteries at their source, the calibers of the vessels that give rise to the collateral supply are small, but the extremely profuse branching and the large size of the derivatives of the bronchial arteries suggest that the resistance to the flow of blood within them is not high. The sum of their cross-sectional areas about any terminal bronchus exceeds that of the pulmonary artery at the same level.

When the precapillary anastomoses have formed, the pulmonary arterial system becomes a huge diverticulum of the circulation now supplied by the bronchial arterial vessels. It may in part absorb the impact of the systemic arterial pressure before the capillaries of the alveoli are reached. Through these large anastomoses, the blood from the bronchial arteries also gains more direct access to the capillaries of the alveoli.

In the present experiments in which the pulmonary artery has been ligated proximally, most of the blood derived from the left side of the heart via the aorta must be returned directly to the left auricle through the pulmonary veins. The burden of the collateral flow therefore falls on the left ventricle. The same is probably true in congenital pulmonic stenosis in which collateral flow may also be extensive,[13] but here the

right ventricle has excessive burdens of its own. In bronchiectasis, however, with much of the alveolar capillary bed obliterated, and with enormous collateral channels that anastomose with the pulmonary arteries,[12] it is possible that blood, entering at the points of anastomosis under systemic pressure, may pass retrogradely toward the hilum in the pulmonary arteries.

Summary and Conclusions

Following ligation of the pulmonary artery, there occurs an enormous expansion of bronchial collateral circulation that is well advanced within 12 weeks. The pulmonary artery does not become thrombosed distally. By 42 weeks, in some animals, precapillary anastomoses with a diameter greater than 50 μ develop between the bronchial and pulmonary circulations. These are associated with bronchioles beyond those of the 6th order in relation to the lobar bronchus. The bronchial arteries associated with the terminal bronchi have a relatively larger total cross-sectional area than the pulmonary artery at the same level. This, together with the absence of microscopic changes in the alveoli, suggests that the peripheral resistance to blood flow in the lungs remains low. The extent of the collateral circulation demonstrated anatomically appears to be compatible with the great magnitude of the flow as determined in physiologic experiments. The collateral flow as observed in these experiments, and as deduced for congenital pulmonic stenosis and bronchiectasis in man, has significant clinical implications in respect to the work of the heart.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

PLATE 27

FIG. 1. Normal dog. Cast of lung viewed from posterior aspect. With the vinylite as employed here, the normal bronchial arteries soon become attenuated below a caliber that will be injected (approximately 50 μ). The right bronchial artery is derived from the 1st right aortic intercostal. The left bronchials, two in number, are derived from the 1st and 2nd right aortic intercostals respectively. The aorta and its branches were injected with black plastic, the pulmonary arteries with red, the pulmonary veins with green, and the bronchial tree with white. The left pulmonary artery is labelled "L" and the interlobar trunk of the right, "R." Approximately two-thirds actual size.

FIG. 2. Dog 15. Sacrificed 3 months after the ligation of the left pulmonary artery. Anterior view of cast. There is a much more extensive plexus of bronchial arteries on the left side. Those on the right are enlarged also, probably on account of their origin in a common trunk with the left. Huge plexuses of vessels are seen astride the left upper lobe bronchus and medially to the left lower lobe stem. The pulmonary veins were injected on both sides, but the pulmonary artery only on the right. Approximately two-thirds actual size.

FIG. 3. Rear view of cast shown in Figure 2. The common origin of the bronchial vessels to both sides is apparent. Very extensive plexuses embrace the bronchi posteriorly on the left, where they can be seen extending to the tips of some of the bronchioles in the lower lobe from which the pulmonary veins have been dissected away. The aorta has been removed. For comparison with Figure 1. Approximately 1½ times actual size.

FIG. 4. Anterior view of injected specimen shown in Figures 2 and 3 before digestion of tissue. The cut distal end of the esophagus has been displaced to the left. Collaterals from the pericardio-phrenic, phrenic, and esophageal vessels are visible. Some of these ramify within the pleura of the left side.

FIG. 5. Dog 33. Sacrificed 10½ months after ligation of the left pulmonary artery. An enlarged intercostal artery is shown extending branches into the left lower lobe. These traversed adhesions before digestion of the tissue. In this instance the entire thorax was dissolved in concentrated hydrochloric acid, thus preserving the relations of the injected vessels. Approximately 1½ times actual size.

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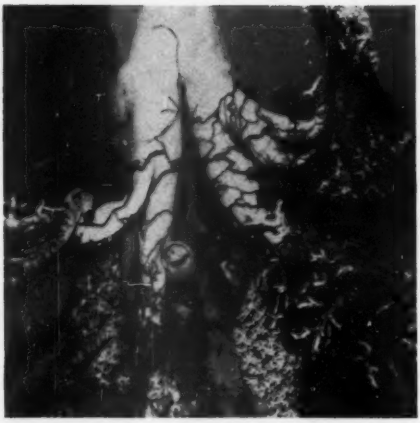

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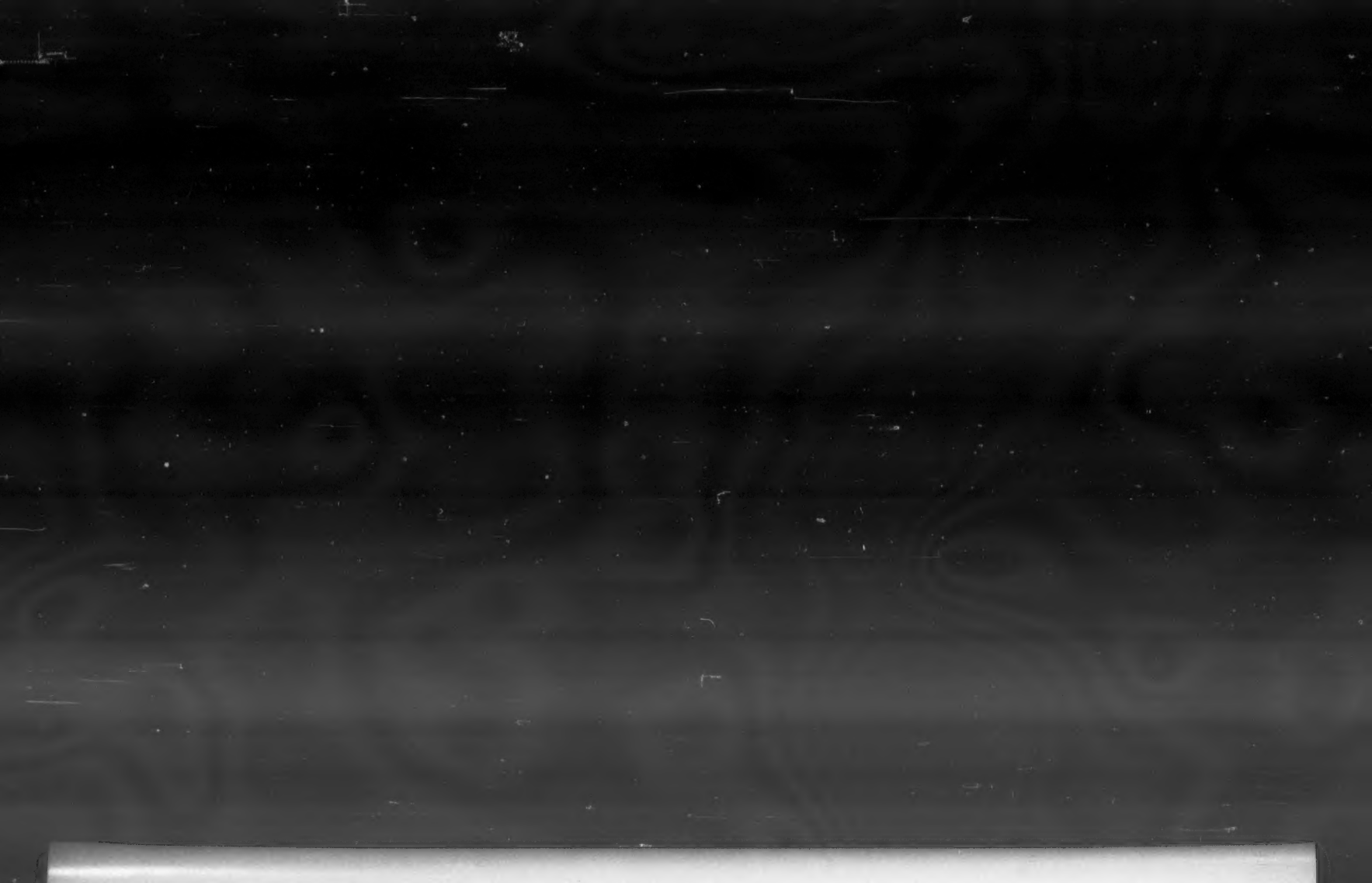
PLATE 28

FIG. 6. Dog 33. A portion of the left upper lobe bronchus and associated vessels is shown. There has been retrograde injection of the pulmonary artery from the enormously enlarged bronchial vessels. The spiraling, many-branched trunks of the latter are more intimately applied to the main bronchus than the pulmonary artery, which faithfully pursues the frond-like distribution of the bronchus. Beyond the 3rd order branches of the lobar bronchus, the comitant main bronchial is only slightly smaller than the pulmonary artery at the same level (see Fig. 13). The exact points of communication of the two systems are not clearly shown in the figure, but are demonstrated in Figure 7. × 2½.

FIG. 7. Dog 33. A peripheral sub-segment of the left upper lobe. The bronchus can be distinguished in faint outline. Its branches are followed by those of a pulmonary arterial trunk (gray, originally in red) which is mottled with the jet black plastic that has entered from the bronchial arteries. The latter are tightly wound about the branches of the bronchus. Their calibers diminish less rapidly than that of the pulmonary artery as they proceed peripherally. In the uppermost branch of the bronchus, the bronchial artery (the tortuous vessel at the right) has nearly the same diameter as the pulmonary artery before it joins it (arrow) via several minute branches located about a bronchus of the 6th order. × 3½.



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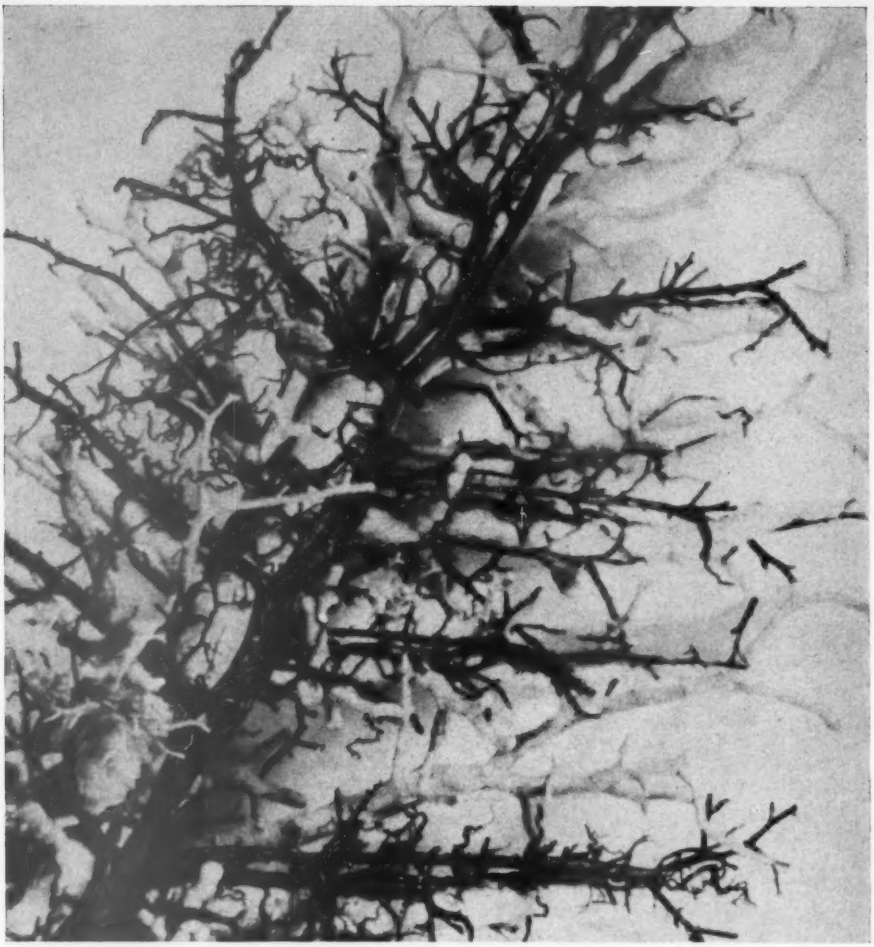

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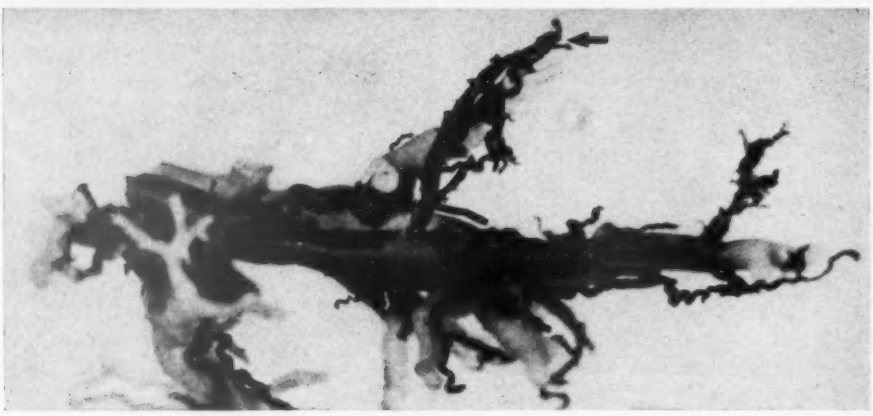

PLATE 29

FIG. 8. Dog 33. An immense plexus of bronchial arteries covers the retrogradely injected pulmonary artery in the interlobar fissure of the left lung. This pulmonary artery had been ligated 10½ months previously. The trunk of the left bronchial artery (arrows) sweeps anteriorly of the aorta from its source in the 3rd right aortic intercostal to supply this plexus. × 2.

FIG. 9. Dog 33 (as in Figs. 5 to 8). Posterior view of cast shown in Figure 8. The greatly enlarged right 3rd intercostal with the 2.5 mm. bronchial trunk (arrow) sweeps downwards from it and then anteriorly of the aorta. The cast has been made by digesting the entire thorax in concentrated HCl after injection. Actual size.

FIG. 10. Dog 11, sacrificed 12½ months after ligation of the left pulmonary artery. Posterior view of cast. The bulbous, ligated end of the cast of the left pulmonary arterial trunk (LP) can be seen just above the upper lobe bronchus. Below it can be seen the distal portion of the left pulmonary artery (LD) which was injected in the same fashion as the trunk. It may be noted that it has the same diameter as the right interlobar branch (R) that is glimpsed between the right upper and lower lobe bronchi. The 1st and 2nd right aortic intercostal vessels are the source of the huge plexus of bronchial arteries that is distributed to the left bronchial tree. (See also Fig. 7.) This plexus contributes only a few small branches to the right lung but sweeps beneath the carina to the left. Actual size.

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PLATE 30

FIG. 11. Dog 56. Right lung. Control. A bronchial arterial branch of the usual size is seen (arrow). Verhoeff's elastic stain. × 18.

FIG. 12. Dog 56. Left lung. Pulmonary artery ligated 7 months previously. Plexus of enormously enlarged bronchial arteries in the walls of the lower lobe bronchus. Vessel marked X is shown under high magnification in Figure 15, Verhoeff's elastic stain. × 18.

FIG. 13. Dog 36. Left lung, 13½ months after ligation of its pulmonary artery. A terminal bronchus approximately 1 by 0.5 mm. in major diameter is shown. The pulmonary artery (P.A.), largely of elastic structure, is seen at the right. More intimately related to the wall of the bronchus are the four lumina of greatly enlarged bronchial arteries, with thick muscular walls. The area of the cross section of the largest bronchial artery in this instance exceeds that of the pulmonary artery. Normally, the bronchial vessels at this level would be represented by arterioles, much smaller than the least of the bronchial arteries seen here. The effective flow of blood through the bronchial collateral vessels in dog 36 was estimated at 1239 cc./M^2/min.[1] × 55.

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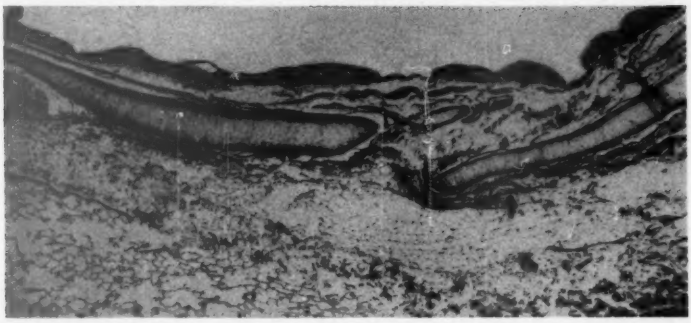

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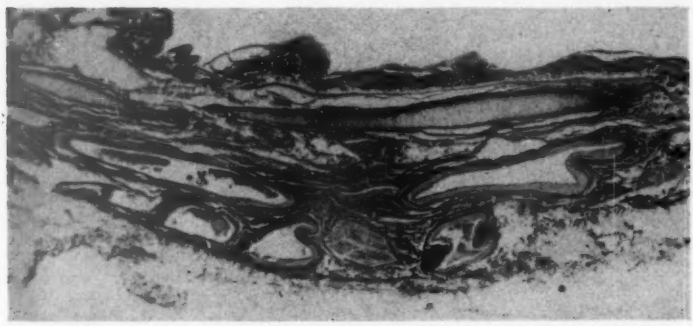

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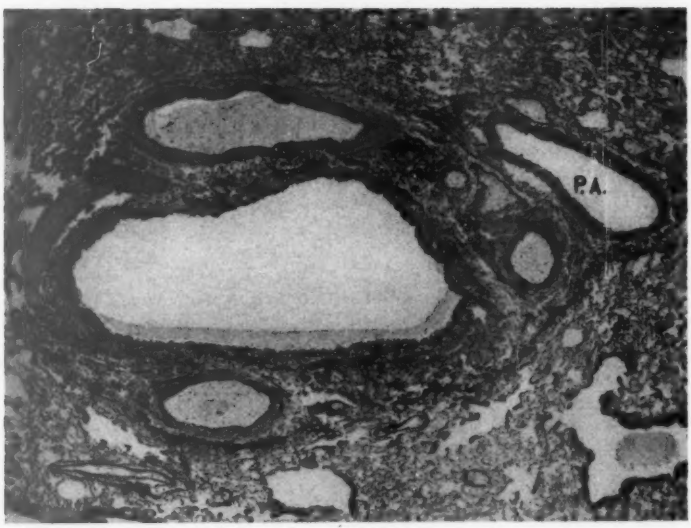

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PLATE 31

FIG. 14. Dog 56. The largest bronchial artery from the wall of the main right upper lobe bronchus of the control lung, enlarged from Figure 11. The internal elastic lamella is a sharply defined continuous band. Most of the wall of the vessel consists of smooth muscle. There are no intimal changes. × 270.

FIG. 15. Dog 56. Left lung. Enlarged bronchial artery shown in Figure 12. Subendothelial pillows of connective tissue. Fragmentation of thickened elastic tissue. Hypertrophy of muscle. × 270.

FIG. 16. Dog 56. Trunk of left pulmonary artery beyond point of ligation showing unaltered, largely elastic, structure. × 270.

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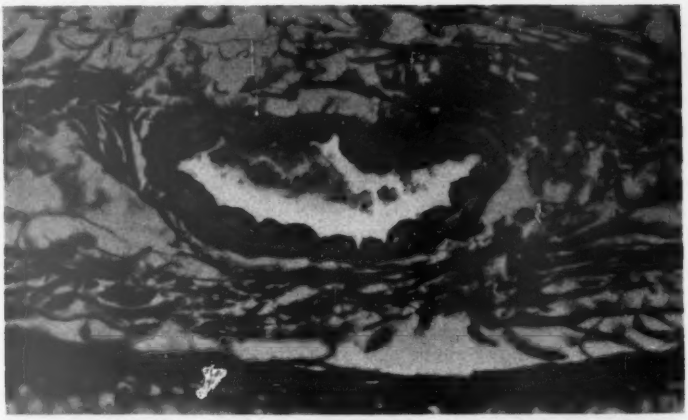

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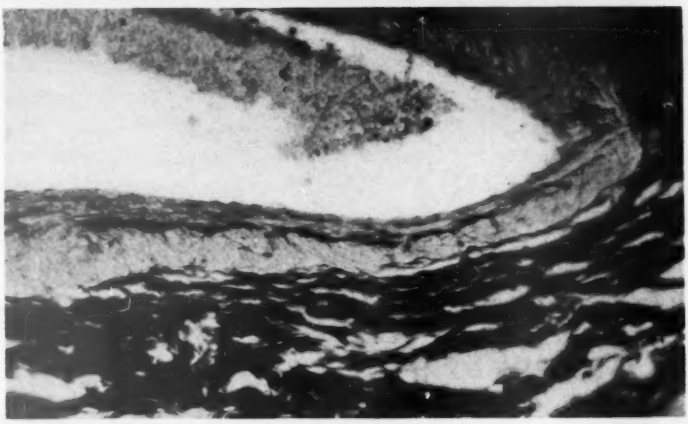

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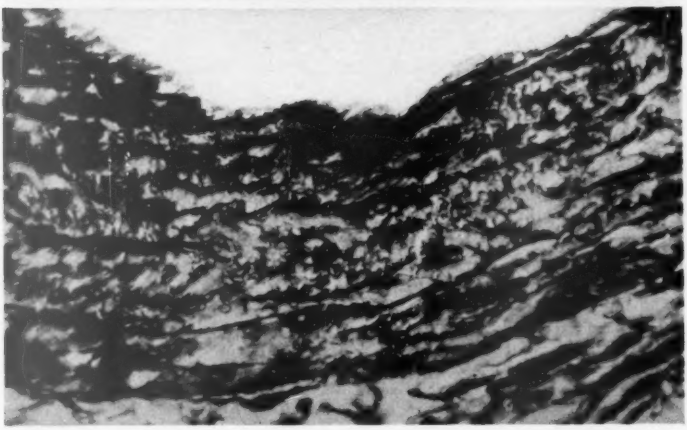

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OSTEOSARCOMA INDUCED BY BERYLLIUM OXIDE*

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During the last 10 years, beryllium has become an increasingly important commercial substance. It is now used in the manufacture of x-ray apparatus, fluorescent lamps, certain types of radio tubes, and fatigue-resistant copper alloys. In its natural state, beryllium is a light metal which is not radioactive.

The possibility that beryllium might be capable of inducing neoplasia was noted by Gardner and Heslington.[1] They found that suspensions of zinc beryllium silicate and of beryllium oxide, given intravenously, induced osteosarcomas in rabbits, but not in guinea-pigs or rats.

This observation was highly significant in view of the fact that only one non-radioactive metallic element (arsenic) has been proved to have carcinogenic properties.

As a part of certain studies of the metabolism and toxicology of beryllium being conducted in this laboratory, an attempt has been made to confirm and extend the work of Gardner and Heslington.[1]

METHODS

Young adult white rabbits, male and female, were used. They were kept in individual cages and were fed stock ration throughout the period of the experiment. It is believed that the experiment was adequately controlled by observations made on approximately 50 rabbits obtained from the same source and kept for similar or longer periods while being utilized for other and unrelated experiments. None of these control animals has developed malignant tumors. Moreover, osteosarcomas have not occurred spontaneously among the large numbers of rabbits observed in this laboratory during prolonged toxicologic investigations over the past 2 decades.

The two materials used consisted of (1) a highly purified beryllium oxide, and (2) a calcined phosphor comprised of beryllium oxide, zinc oxide, and silica mixed in the molar ratio of 1:1:1. Neither of these materials was radioactive, as shown by negative tests with the Geiger-Müller counter and by autoradiographs. The mean particle size of each powder was found by electron microscopy to be below 1 μ, and the powders were administered as 1 per cent suspensions in physiologic saline solution. The beryllium oxide was found to be only slightly

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soluble. One gram of beryllium oxide powder was suspended in a liter of distilled water, and 1 week after making the suspension the concentration of beryllium in the solution was 0.7 μg. per 100 cc.

An injection was made into an ear vein of each animal three times per week until the intended amount of material had been administered. The number of doses per animal ranged from 17 to 26, and the amounts of beryllium (calculated as metal) ranged from 0.013 gm. per kg. of body weight to 0.116 gm. per kg. of body weight (Table I).

TABLE I

Osteosarcomas Induced by Beryllium

Rabbit no.	Substance (1% suspension in saline solution)	Dose	Number of doses	Total amount of Be	Date of first dose	Date of last dose	Date tumor found
		cc.		*gm.*			
Be 17	Phosphor	8	21	0.09	8–14–47	10– 3–47	8–16–48
Be 24	BeO	8	23	0.66	8–14–47	10– 6–47	10–16–48
Be 26	BeO	5	20	0.36	9–15–47	11– 3–47	8–27–48
Be 29	BeO	7.5	26	0.70	9–15–47	11–15–47	9–14–48
Be 31	Phosphor	8	25	0.08	9–17–47	11–15–47	9– 2–48
Be 27	BeO	5	20	0.36	9–15–47	11–15–47	10–13–48
Be 4	Phosphor	7	17	0.064	8–27–47	10– 6–47	*
Be 23	BeO	6–7	21	0.50	8–14–47	10– 3–47	†
Be 28	BeO	7.5	24	0.58	9–15–47	11–15–47	†

* No tumors found; observations being continued.

RESULTS

After the animals had been given their final injection, they were examined periodically by palpation of the skeletal structures for the presence of tumors. Osteosarcomas developed in 6 of the 9 animals that lived for 1 year or more after the first injection. The first tumor noted appeared 11½ months after the start of the experiment.

Post-mortem examination was required to reveal the tumors in 3 of the animals, even though progressive loss of weight in the weeks immediately prior to death indicated that tumors might be present. In one of these, the location of the primary tumor in the vertebral column was suspected prior to death when the animal developed signs which were consistent with compression of the spinal cord by tumor (paralysis of the hind limbs and loss of control of anal sphincter). In the other animals, the neoplastic growth was discovered by palpation.

† Since this paper was submitted for publication, these 2 rabbits have died of osteogenic sarcoma with pulmonary metastases.

In 4 rabbits, single primary tumors were found. These tumors were located in the right scapula, the head of the right humerus, the body of the last lumbar vertebra, and the lower end of the right femur. Metastatic tumors were found in the lungs of all 4 of these animals, and in addition there were metastatic nodules in the parietal pericardium, the parietal pleura, and the liver of one of these animals.

There were multiple primary tumors in each of the other 2 rabbits. One of them had an osteogenic sarcoma of the upper end of the right tibia, and a similar primary tumor in the head of the right humerus. In this animal there were masses of metastatic tumor in the lungs and the liver. The second animal had five primary centers of neoplastic growth, and there were metastases in the lungs. The primary tumors were in the lower end of the right humerus, the upper end of the left humerus, the body of one of the anterior lumbar vertebrae, the upper end of the left tibia, and the upper end of the right tibia.

Some primary tumors had broken through the cortical bones and were invading the adjacent muscles (Figs. 1 and 2). These invading tumors were surrounded by pseudo-capsules of fibrous tissue in which degenerating muscle fibers were frequently visible microscopically. The largest primary tumor, arising in the right scapula, was 11 by 9.4 by 6.2 cm. (Fig. 3). In some of the animals the primary tumors were found only after complete dissection of the skeleton with splitting of all bones, and in several instances the tumors had not extended through the cortical bone. In all cases, the fact that tumor was present was confirmed microscopically.

The cut surfaces of the larger tumors were mottled with small yellowish orange necrotic areas and in some places there were foci of recent or partially decolorized hemorrhage. In the 2 cases in which primary tumor was found in the bodies of vertebrae, the tumor had extended through the cortex of the vertebral body and was pressing against, but not invading, the spinal dura mater and the spinal cord.

The metastatic tumors in the lungs consisted grossly of subpleural and intraparenchymal nodules, ranging up to 1.2 cm. in diameter. The larger subpleural nodules tended to be slightly umbilicated. Nearly all of the nodules were firm, and spicules of bone were encountered by the sectioning knife. Some lungs contained only a few small nodules, while in other animals as much as one-half of the total volume of lung tissue was occupied by tumor nodules (Fig. 4). Metastatic nodules in the parietal pleurae, pericardium, and liver were like those in the lungs.

There was focal fibrosis of bone marrow in the ends of some of the long bones in all animals. In one animal the head of the right humerus was occupied by a tumor which was contiguous with dense fibrous

tissue in the neck of the bone, while the body of the humerus was filled with red marrow. In all of the animals, the red marrow contained numerous white flecks which, microscopically, were found to be collections of foreign material (beryllium oxide or phosphor dust) in phagocytic cells. Similar white flecks were seen also in the spleens of the animals, and microscopically the material was found to be within phagocytic cells. The spleen of one animal was grossly fibrotic, and increased fibrous tissue was found in the others by microscopy. In one animal the liver was grossly normal, but in the others there was extensive diffuse fibrosis and cirrhosis, and large areas of liver tissue in some of the animals were completely replaced by dense fibrous tissue.

The microscopic structure of all of the tumors was that of typical osteosarcoma. Several types of tissue could be identified in each tumor (Figs. 5 and 6). There were regions of large, poorly differentiated cells (Fig. 8), and there were other regions where the tissue was made up of atypical, partially differentiated fibroblasts (Fig. 7). Still other areas were comprised of atypical cartilage or of osseous tissue (Figs. 5 and 6).

Large vascular spaces were numerous in the tumors. The endothelial lining was incomplete in some of these spaces so that malignant tumor cells lined the vessels in such regions (Fig. 10). Masses of tumor cells could be seen proliferating within the lumina of vessels in some of the primary tumors (Fig. 9), and in all animals neoplastic emboli were present in the pulmonary arteries (Fig. 11).

None of the tumors contained visible particles of the foreign materials which had been injected, and the quantities of beryllium recovered from the tumors by analysis were small. In an effort to determine if this tumor were capable of existing in a beryllium-free environment, bits of tumor from one rabbit were transplanted to one of the anterior chambers of several guinea-pigs, in the manner described by Greene.[2] It was found that the tumor continued to grow in the eyes of these animals.

Tissues of the animals which developed tumors were analyzed for beryllium (Table II). The liver and spleen of each of the animals contained relatively large quantities of beryllium, presumably as a consequence of the functional activity of the reticulo-endothelial cells in these organs. The quantities found in the lungs may represent aggregates of particles which were filtered out by the capillary bed of the lungs during and immediately after the intravenous injections.

Entire long bones which were not involved by tumor were analyzed. Microscopic studies of such bones have revealed that most of the beryl-

lium is probably contained in the reticulo-endothelial cells of the marrow. The quantities of particulate material visible in the littoral cells of the marrow were comparable to those seen in the sections of liver and spleen, and presumably, if the marrow had been analyzed alone

TABLE II

Beryllium in Tumors and Other Tissues
Milligrams of Beryllium per 100 Grams of Tissue

Rabbit no.	Primary tumor	Bone without tumor	Lung (including metastatic tumor)	Liver	Spleen	Kidney	Heart	Urine
Be 17	0.0007	0.305	21.8	9.1	64	0.50	0.793	
Be 24	*	4.90	39.5	225		0.41		0.0054
Be 26	0.093	0.308	133	184	235	4.92	1.64	
Be 29	*	0.146	22.2	5.9	75	1.08	0.722	
Be 31	0.035	0.213		8.7	136	0.81		0.004
Be 27	0.038	0.430	43.7	36.3	320	1.99	3.43	0.114

* Tumor too small for analysis.

without cortical bone, the concentrations of beryllium found therein would have been of the order of magnitude found in the spleens and livers. The kidneys and hearts of these animals contained relatively little beryllium, probably because there are few reticulo-endothelial cells in these organs. The amounts found in the primary tumors were minute.

DISCUSSION

The ability of simple beryllium compounds (beryllium oxide, and a calcined mixture of beryllium oxide, zinc oxide, and silica) to stimulate neoplastic growth has been confirmed. The manner in which beryllium is capable of altering normal cells so that they become malignant is unknown. It is recognized that beryllium is capable of inducing proliferation of fibrous stroma in the tissues of man[3] and experimental animals,[4] and it is conceivable that some fundamentally similar process is responsible for the induction of neoplastic proliferation. In the animals which developed malignant tumors, there was, in addition, fibrosis in the livers and spleens, as well as some foci of fibrosis in the bone marrow. In the regions of fibrosis, collections of the irritant dust could be seen in the littoral cells.

That these osteogenic sarcomas could become independent of beryllium after they had begun their development was suggested when only minute amounts of beryllium were found by analyses of them. Further evidence on this score was provided by the successful transplantation

of tumor fragments to the anterior chambers of the eyes of guinea-pigs in which they continued to grow.

At present, there is no reason to believe that the fibrosis which occurs in human berylliosis is likely to lead to the development of tumors. There is no evidence indicating that any tumor has been induced by beryllium compounds except after their intravenous administration to rabbits.

SUMMARY

Malignant bone tumors (osteogenic sarcomas) have developed in 6 of 9 rabbits within the period of 16 months after the beginning of serial intravenous injections of beryllium oxide or of a phosphor containing beryllium oxide. The injections were given three times a week and were continued for from 6 to 8 weeks.

The primary tumors, when subjected to chemical analysis, have been found to contain little beryllium. Tumor fragments from one animal were capable of growth in the anterior chamber of the eyes of guinea-pigs. Thus, the continued growth of the osteogenic sarcomas, once established, appears to be independent of the presence of beryllium.

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DESCRIPTION OF PLATES

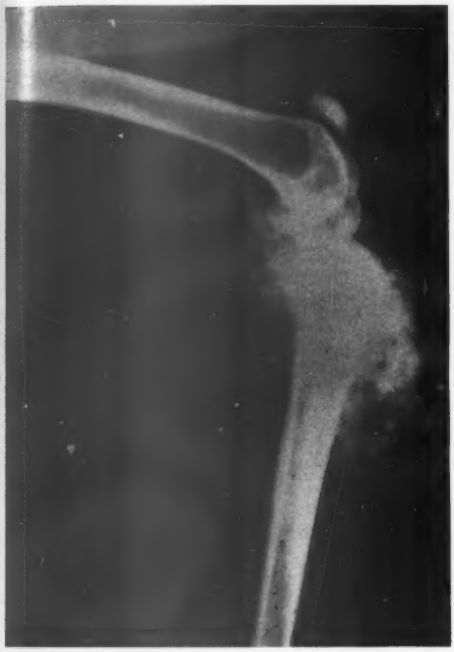
PLATE 32

FIG. 1. Rabbit 31. Roentgenogram. Primary osteosarcoma of tibia induced by phosphor containing beryllium oxide.

FIG. 2. Rabbit 26. Primary osteosarcoma of lower end of femur. There is invasion of the skeletal muscle, and hemorrhagic and necrotic areas may be noted in the tumor.

FIG. 3. Rabbit 17. Primary osteosarcoma arising in scapula, with invasion of adjacent muscles. Necrotic areas and hemorrhages are extensive. The glenoid fossa is at the upper right.

FIG. 4. Rabbit 17. Metastatic osteosarcoma of lungs.



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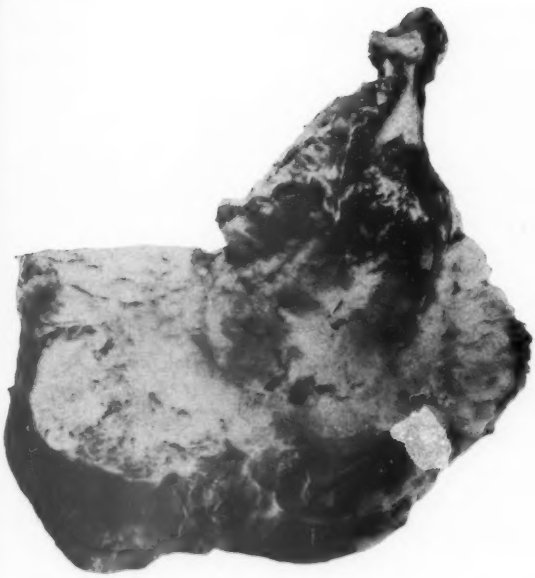

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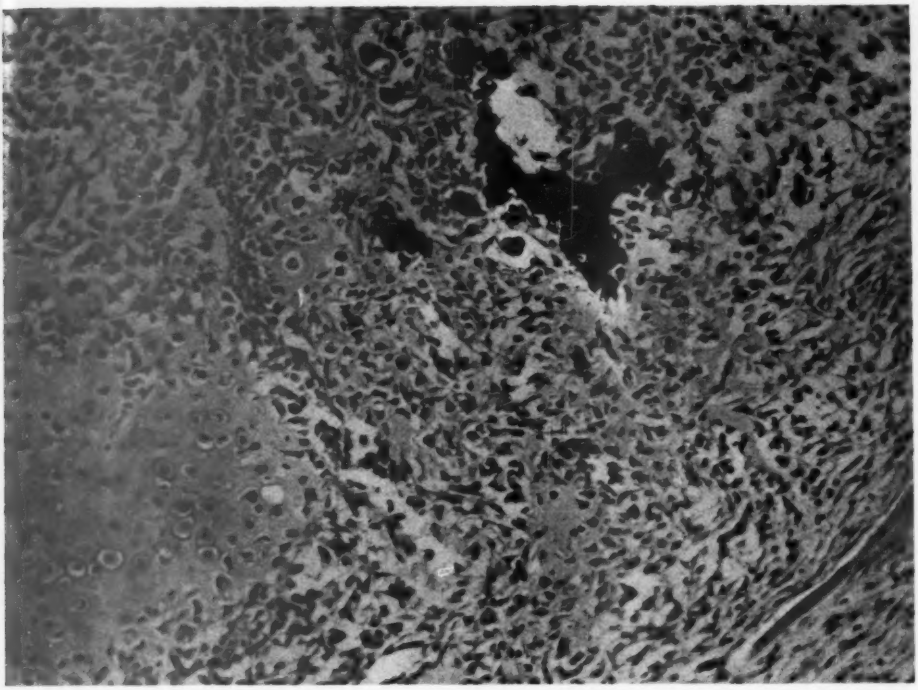
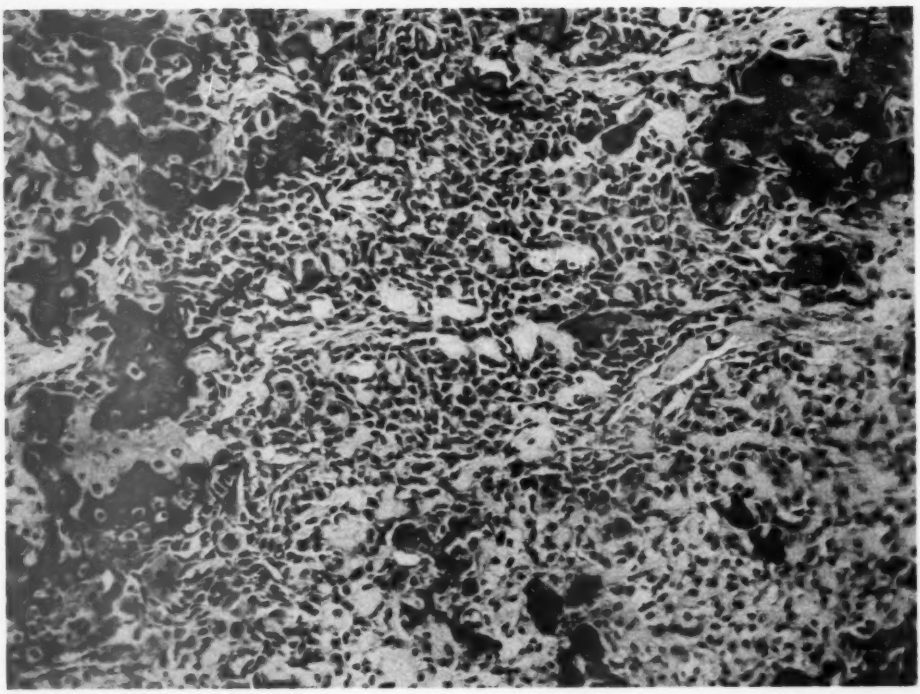
PLATE 33

FIG. 5. Rabbit 31. Tissue taken for biopsy of a primary osteosarcoma of the tibia, showing heterogeneous nature of the structure of the tumor. Abnormal cartilage, with focal calcification, and poorly differentiated tumor cells are seen. A fibrous pseudo-capsule in which there is a degenerating skeletal muscle fiber is included in the lower right corner. × 160.

FIG. 6. Rabbit 31. Another region in the excised tissue used for Figure 5, in which fragments of osseous tissue are numerous. × 160.



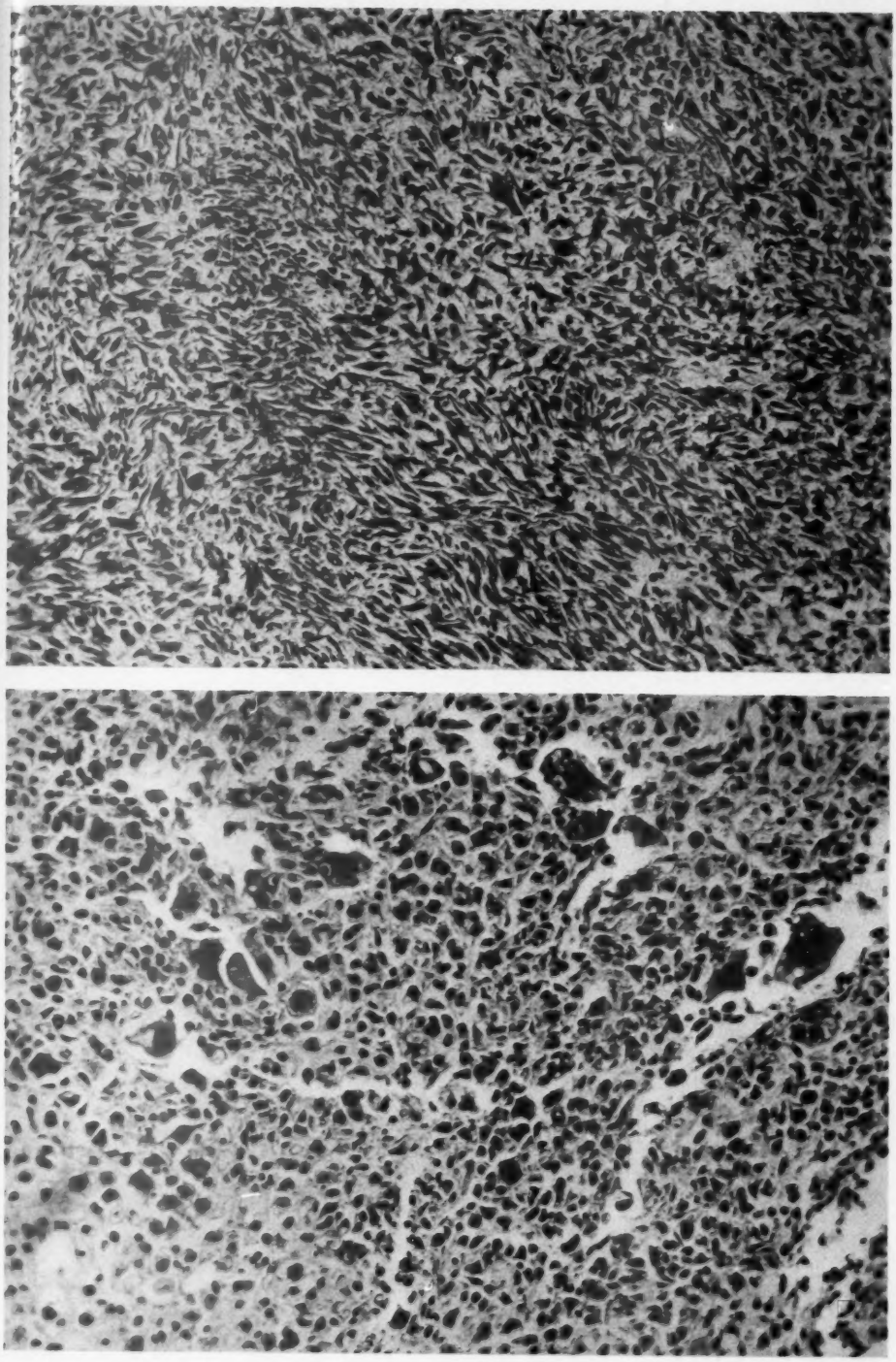


Dutra and Largent Osteosarcoma Induced by Beryllium Oxide

PLATE 34

FIG. 7. Rabbit 29. Region of poorly differentiated spindle and polygonal cells, some multinucleated, from a primary osteosarcoma. × 160.

FIG. 8. Rabbit 29. Another area of poorly differentiated tumor, in which there are numerous giant cells. × 160.



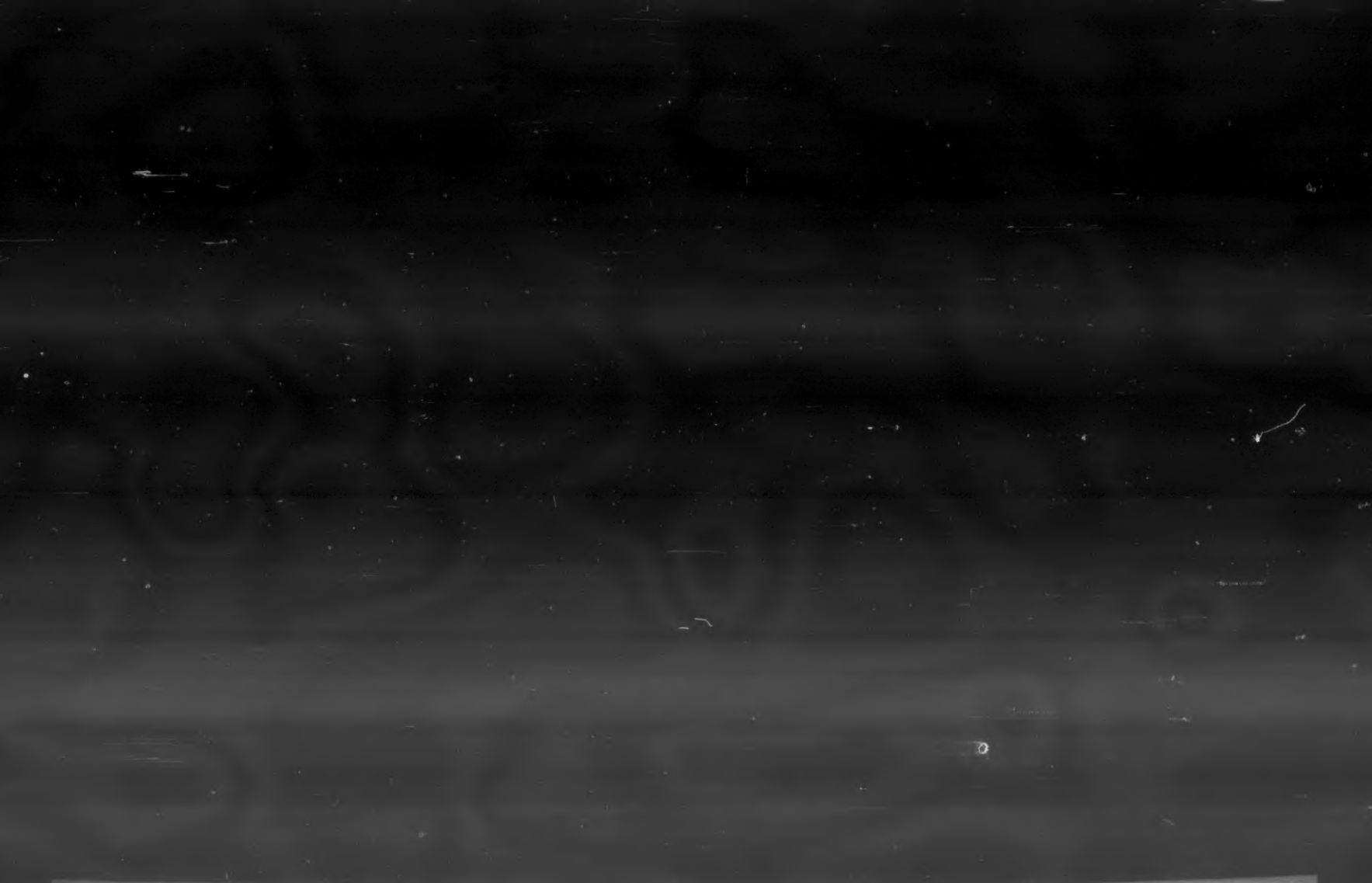
Dutra and Largent Osteosarcoma Induced by Beryllium Oxide

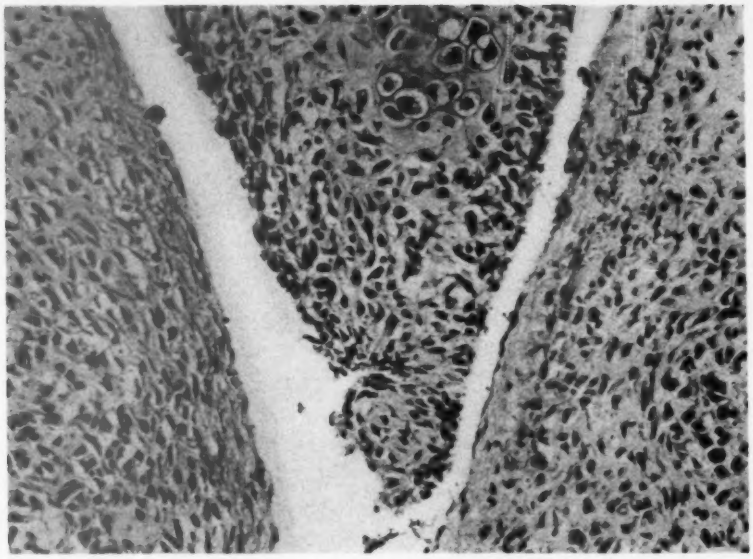
PLATE 35

FIG. 9. Rabbit 26. A large vein surrounded by tumor cells in a primary osteosarcoma in lower end of femur. In the lumen of the vessel is a mass of viable neoplastic tissue, an example of tumor spreading within vascular channels, and a ready source of embolic metastasis. × 160.

FIG. 10. Rabbit 26. Two large vascular channels in a primary osteosarcoma of the femur. The endothelial lining of each is fragmentary and tumor cells project into the lumina. × 150.

FIG. 11. Rabbit 26. A mass of metastatic osteosarcoma lodged in a pulmonary artery. The tumor is comprised of abnormal cartilage in which there is calcification. Invasion of the arterial wall is visible at the margin of the embolus toward the right. × 150.





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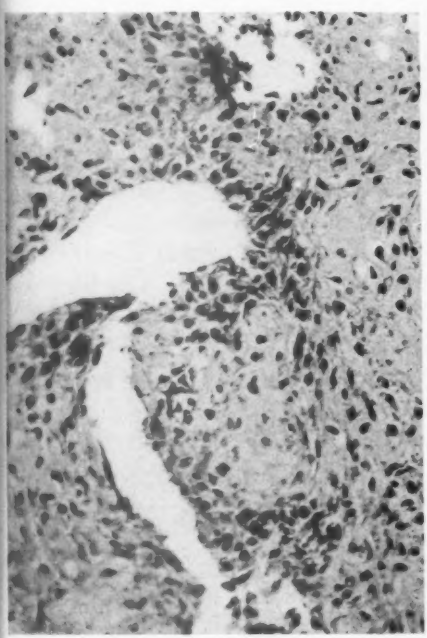

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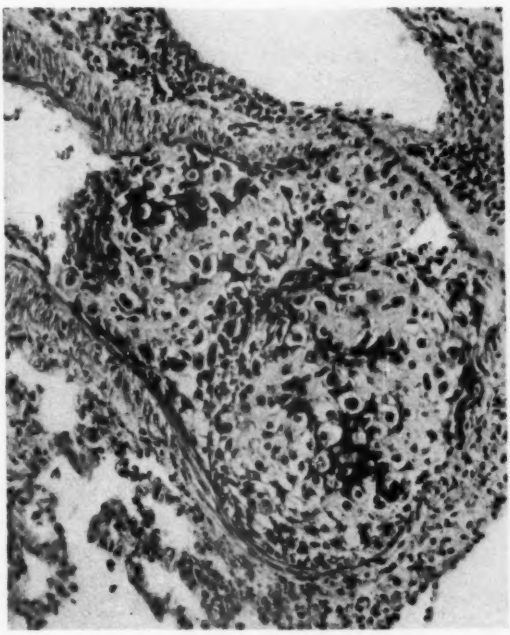

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Osteosarcoma Induced by Beryllium Oxide

HORMONALLY INDUCED TRANSFORMATION OF ADRENAL INTO MYELOID TISSUE *

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Many observations suggest some intimate relationship between the adrenal cortex and hemopoiesis. The anemia of Addison's disease, the polycythemia frequently associated with hypercorticoidism (adrenal cortical hyperplasia, Cushing's syndrome, and adrenal tumors), as well as several case reports recording the appearance of myeloid or lymphatic tissue in the adrenal, all intimate that close, though little understood, correlations may exist between blood cell formation and the suprarenal glands.

We became interested in this subject in 1934 (McEuen and Selye[1,2]) when, in the adrenals of rats bearing the Walker rat tumor, we noted the frequent appearance of islets, consisting of lymphocytes and polymorphonuclear leukocytes. These islets were particularly constant and numerous in rats whose tumor transplant contained large areas of necrosis. They were therefore tentatively ascribed to toxic products of decomposition of tissue, absorbed from liquefied necrotic areas of the neoplasm. This interpretation received support from subsequent experiments in which we were able to produce similar adrenal changes by the injection of cell-free extracts of necrotic tumor. Since even repeated intraperitoneal transplantation of sterile rat kidney induced such islets in the adrenals, we concluded that during decomposition, not only neoplastic, but even normal cells can give rise to toxic products which cause such morphologic changes in the adrenals. It was noted also in the course of this work that simultaneously with the adrenal islets, rather typical morphologic changes appeared in the anterior pituitary gland. These were always the same irrespective of the technic used to produce the islets (tumor transplants, tumor extracts, or kidney implants) (McEuen, Selye, and Thomson,[3] McEuen and Selye[1,2]).

Later it was observed that impure, corticotrophin-containing, bovine anterior pituitary extracts are particularly active in producing leukocytic islets in the adrenal of the rat.[4] Sometimes severe overdosage with these preparations resulted in the formation of polymorphonuclear giant cells within the adrenal cortex, and these bore a striking resemblance to the megakaryocytes of bone marrow.[5]

In the light of these observations, it was reasonable to assume that the corticotrophic action of the anterior pituitary body may play a rôle

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in stimulating hemopoiesis in the adrenal. However, this interpretation was difficult to reconcile with the well known fact that injection of pure corticotrophin or endogenous overproduction of this hormone, as seen during the general adaptation syndrome,[6] does not stimulate hemopoiesis in suprarenal tissue. We were led to believe, therefore, that both corticotrophin and toxic products of tissue decomposition are necessary to induce this peculiar change.

It remained somewhat doubtful whether these islets should be interpreted as true bone marrow tissue since they completely lack an organoid character. In particular, they are devoid of a fat-cell-containing reticular stroma so characteristic of normal, fully differentiated, adult bone marrow. Thus, these foci were more reminiscent of the infiltrations which appear in various tissues during certain leukemias in man, or of the erythropoietic nodules, which have been produced experimentally in splenectomized guinea-pigs rendered anemic with phenylhydrazine (Hayashi[7]).

Our subsequent investigations showed that in the rat, testoid compounds, especially methyl testosterone, cause the appearance in the adrenal cortex of extraordinarily large vacuoles containing sudanophilic lipid material. Often a single large lipid granule distended the body of the adrenal cortical cell to such an extent as to push the nucleus to one side, thus resulting in signet-ring types similar to the fat cells of ordinary adipose tissue (Selye, Rowley, and Hall[8]). Here again it was tempting to assume that the change is mediated by the production of pituitary corticotrophin, especially since in hypophysectomized animals testosterone did not exert this effect. However, even very chronic overdosage with large amounts of corticotrophin failed to produce such signet-ring cells in the adrenal, although the glands became extremely large and laden with numerous small lipid granules. We consequently concluded that perhaps this transformation of adrenal cortical cells into "fat cells" was due to the simultaneous action of corticotrophin and testoid hormones, just as the production of hemopoietic foci was dependent upon both corticotrophin and the products of tissue necrosis.

The experiments reported in the present communication were designed to study the effect of synchronous treatment with LAP, a crude anterior pituitary preparation (known to produce myeloid foci) and of testoids (known to cause transformation of adrenal cortical cells into "fat cells").

Materials

Experimental Animals and Their Diet. For these experiments, 42 adult female albino rats were used. The initial body weight was between

145 and 155 gm., with an average of 149 gm. Since the diet greatly influences corticotrophic responses,[4] it is necessary to state that all of our animals were given a synthetic diet (Table I).

The experimental animals were unilaterally nephrectomized the day before the initiation of hormone treatment. Subsequent observations

TABLE I

Composition of the Basic Synthetic Diet

Diet (parts per cent)	
Casein	18.0
Cornstarch	74.0
Fat (mazola)	1.0
Cod-liver oil	1.0
Celluflour	1.0
Mineral mixture	4.0
Supplements	0.54
Supplements, 0.54 gm. per 100 gm. of diet	
Thiamine chloride	0.8 mg.
Riboflavin	0.8 mg.
Pyridoxine	0.8 mg.
Ca pantothenate	1.5 mg.
Nicotinic acid	1.5 mg.
Choline chloride	400.0 mg.
Inositol	100.0 mg.
p-amino-benzoic acid	30.0 mg.
Mineral mixture, 4 gm. per 100 gm. of diet	
NaCl	23.4 gm.
$MgSO_4 \cdot 7H_2O$	24.6 gm.
Na_2HPO_4	14.2 gm.
K_2HPO_4	69.6 gm.
$CaHPO_4 \cdot 2H_2O$	69.8 gm.
Ca lactate $\cdot 5H_2O$	15.4 gm.
Ferric citrate	1.2 gm.
KI	0.16 gm.

revealed that unilateral nephrectomy does not significantly influence the adrenal reaction which we wish to describe in the present communication, but in this particular series we also wanted to examine the influence of hormone treatment upon the organ changes produced by adrenal stimulation; our previous work along these lines[4] had shown that unilateral nephrectomy sensitizes the rat to the toxic manifestations of increased corticoid hormone production.

The animals were subdivided into 7 groups, each consisting of 6 rats. Group I rats were intact controls. Groups II to VII were unilaterally nephrectomized. Group II received no hormone treatment, group III was treated with methyl testosterone, group IV with thyroxin, group V with LAP, group VI with methyl testosterone and thyroxin, group VII with methyl testosterone, thyroxin, and LAP.

On the 57th day of treatment all animals were killed and their adrenals removed for histologic study.

The hormone preparations used were prepared and administered as follows:

Lyophilized Anterior Pituitary (LAP). Lyophilized anterior pituitary tissue was prepared from fresh beef adenohypophyses, by suspending 40 mg. of dry powder per cc. of saline solution. This was administered subcutaneously twice daily in an amount equivalent to 15 mg. Since very prolonged treatment with LAP is poorly tolerated, injection of this material was commenced only on the 35th day of the experiment and continued during the last 22 days of observation.

Methyl Testosterone. Crystalline synthetic methyl testosterone was compressed into pellets, each weighing approximately 50 mg. Two of these pellets were implanted subcutaneously, one at the beginning of the experiment, the other on the 35th day of treatment.

Sodium Thyroxinate. A microcrystalline suspension, containing 500 μg. per cc. of sodium thyroxinate in distilled water, was given in doses of 0.1 cc. once daily, subcutaneously. We used sodium thyroxinate in some groups, since our previous experiments had shown that thyroxin increases the corticotrophic effect of anterior pituitary preparations (Selye, Stone, Nielsen, and Leblond[9]).

Observations

Histologic study of the adrenals revealed that methyl testosterone (group III) caused the deposition of large lipid accumulations within the cytoplasm, eventually resulting in the transformation of the adrenal cortical cells into elements exhibiting the characteristics of ordinary fat cells. Thyroxin (group IV) caused some hypertrophy and hyperplasia of the adrenal cortex without any very distinctive specific characteristics, while simultaneous treatment with methyl testosterone and thyroxin (group VI) merely resulted in a slight accentuation of the fat cell formation seen in animals treated only with methyl testosterone (group III).

The rats receiving LAP alone (group V) showed moderate signs of myeloid transformation in the adrenal cortex (Figures 1 to 3). This process was essentially the same as that which we previously noted under similar conditions (Selye[4]). In certain regions of the cortex and, to a lesser extent in the medulla, cells appeared which exhibited the characteristics of normal hemopoietic tissue. Figure 1 illustrates two such foci in the adrenal cortex, as seen at a low magnification.

Upon inspection under high magnification it became evident that this hemopoietic tissue appears to result—at least in part—from the direct metaplasia of typical adrenal cortical cells into hemopoietic cells. All

transitional stages could be identified between normal, lipid-granule-containing large cortical cells with their regular round nuclei and well developed cytoplasm, and lymphocytes, polymorphonuclear leukocytes, myeloblasts, myelocytes, and normoblasts, such as are normally found in bone marrow. Occasionally, even megakaryocyte-like, large, polynuclear giant cells were observed to arise by fusion of several adrenal cortical cells or by atypical multipolar mitosis (Figs. 2, 3, and 4).

It should be emphasized that, although in many regions the direct metaplasia of the cortical cell into the hemopoietic tissue was evident, this does not preclude the possibility of hemopoiesis from reticulo-endothelial cells in the same adrenal. Indeed, in some sections, the proliferation of reticulo-endothelial cells was highly suggestive of simultaneous hemopoiesis from these latter elements. The most outstanding difference between this ectopic hemopoietic tissue and that normally found in the bone marrow was the complete absence, in the former, of fat cells so characteristic of bone marrow stroma.

In view of the fat cell formation in animals treated with methyl testosterone, it was particularly instructive to study the adrenals of the animals in group VII, which were simultaneously treated with LAP (the effects of which were synergized by thyroxin, and with methyl testosterone. In this group, a most unusual type of adrenocortical metaplasia occurred. The entire zona reticularis and inner fasciculata region were completely transformed into apparently typical bone marrow tissue; the outer fasciculata and glomerulosa layers remained normal although exhibiting some hypertrophy and hyperplasia such as is usually found following treatment with corticotrophic anterior lobe extracts. The medulla contained occasional islets of round cell infiltration, but otherwise retained its normal aspect. As a result of this transformation, on cross sections through the central part of the adrenal we noted a medulla surrounded by a circular layer of bone marrow tissue, which in turn was enveloped by normal adrenal cortical parenchyma. This same, clearly demarcated transformation of the inner cortical layers was noted in all experimental animals of group VII (Figs. 5 and 6). The impression was gained that metaplasia of the inner cortical layers into "adipose tissue" by methyl testosterone had provided a favorable medium for the development of myeloid cells and enhanced the organoid, bone-marrow-like appearance of the tissue in this region.

Figure 7 represents a general view of the adrenal cortex in one of these experimental animals, which permits direct comparison with a similar case seen in man (Collins[10]), and reproduced as Figure 8 at the same magnification.

It is important to emphasize that although myeloid metaplasia was most pronounced in the adrenal cortex, foci of hemopoietic tissue have appeared also in some other tissues of these rats. A particularly common location for them was the so-called brown fat or hibernating gland found in the region of the renal pelvis of the rat. It is perhaps significant that the cells of the brown fat bear a striking resemblance to those of the adrenal cortex; they are large polygonal elements containing numerous small granules and many mitochondria. It is also noteworthy that the brown fat cells, like those of the adrenal cortex, tend to discharge their lipid granules during exposure to general stress such as is conducive to an alarm reaction (Selye[4]).

The fact that granulomatous infiltrations occur with extraordinary frequency in the region of the renal pelvis of rats treated with crude anterior pituitary extracts has been mentioned in one of our earlier publications (Selye *et al.*[9]), but at that time only superficial attention was given to this lesion and we did not recognize it as part of a general myeloid response.

In the present experiments we carefully followed the transformation of these brown fat cells into hemopoietic tissue and noted (as in the case of the adrenal cortex) that it is, at least in part, presumably due to the direct transformation of the original cells into hemopoietic elements. Many transitional stages are seen between the brown fat cell and the various bone marrow elements, and eventually the whole brown fat cell nodule is completely replaced by hemopoietic tissue (Figs. 9 to 11). Even typical megakaryocytes, similar to those normally seen in the bone marrow and frequently observed in the adrenal cortices of our rats, were commonly noted in the brown fat tissue region (Figs. 12 to 14).

The most pronounced changes occurred in the spleen, whose weight was almost three times the normal in group VII. Upon histologic examination this increase was shown to result from an intense proliferation of both lymphatic and myeloid elements. Among the latter, extraordinarily large megakaryocytes in striking number were especially prominent even at a very low magnification. These giant megakaryocytes were particularly common in the region immediately surrounding the lymphatic follicles of the spleen.

Similar changes were frequently noted also in the stroma of the liver, kidney, and other organs, but in them they were much less striking than in the adrenals, the brown fat, and the spleen. The proliferation of hemopoietic elements in the bone marrow itself was likewise demon-

strable in many experiments of this kind but we did not study it as closely as the ectopic hemopoietic tissue accumulations.

Discussion

In attempting an interpretation of our findings in terms of human pathology, it is interesting to survey them in relation to the literature concerning similar changes, which occur spontaneously in man.

Fat Cells in the Adrenals. The stroma of the human adrenal frequently contains ordinary fat cells (Kiyokawa[11]), and there may be transitional types between these and the adrenal cortical cells with comparatively large lipid granules. Gossmann[12] published a particularly detailed study concerning this subject and gave excellent reproductions showing isolated signet-ring cells in the midst of the normal cortical parenchyma. He pointed out that such fat-cell-like elements are never found in children and become increasingly more frequent with age. According to this author, atherosclerosis and various nephropathies predispose the individual to the development of this heavily lipid-laden cell type.

An increase in the size and number of the adrenal cortical lipid granules is commonly seen in animals adapted to some kind of systemic stress as well as in men suffering from hypertension, nephrosclerosis, arteriosclerosis, and other diseases of adaptation (Gossmann,[12] Duthoit,[13] Chauffard, Laroche, and Grigaut,[14,15] Weltmann,[16] Borberg,[17] Dietrich and Siegmund[18]). It is a typical manifestation of the stage of resistance of the general adaptation syndrome[6] and presumably represents a corollary of hormone storage. Actual formation of fat cells may merely be an exaggerated and abnormal form of this same process. It is possible, though yet unproved, that stimuli other than the testoid hormones can so exaggerate fat storage in the adrenal cortex as to cause adipose metaplasia.

Round Cell Infiltrations and Lymphatic Nodules. Small foci containing round cells with little cytoplasm have often been noted in the adrenals of man. They are especially common in the so-called primary contracted adrenal which is frequently conducive to Addison's disease (Wiesel,[19] Selye[4]), but also are seen in connection with a great variety of other diseases, usually being noted as an incidental finding at autopsy. They have been described as round cell infiltrations (Costa,[20] Cavallero,[21] Moretti[22]), or foci of lymphocytes (Moretti,[23] Kolmer[24]); Wiesel[19] expressed the view that they are sympathoblasts (!) while Costa[25] regarded them as a special cell type *sui generis*, which has

nothing to do with the primordia of the sympathetic ganglion cells or the parenchyma of the adrenal cortex and medulla. Bittorf[26] stated that round cell infiltration islets are particularly common in the adrenals of patients who died from carcinoma, nephritis, or arteriosclerosis, which again suggests some relationship to the diseases of adaptation. Sala and Stein[27] came to the conclusion that "The presence of leukocytes in great numbers in the cortex and medulla of the adrenals of patients dead of neoplastic disease is not a specific finding in such disease, but a factor which is apparently present whenever extensive necrosis of tumor tissue occurs." They considered their observations to support our previously mentioned animal experiments with the Walker rat tumor.

Small cell infiltrations in both cortex and medulla are likewise frequently observed in chronic nephritis and other conditions combined with hypertension (Thomas,[28] Bittorf[26]) although these changes are not characteristic of these diseases and may also be observed in chronic infections (d'Alessandro[29]).

The intense infiltration of the adrenals with polymorphonuclear leukocytes which Davis[30] noted in experimental dehydration shock and Ferguson and Chapman[31] in the Waterhouse-Friderichsen syndrome, is probably a related phenomenon.

Bone Marrow Tissue in the Adrenal. The formation of erythropoietic foci (containing myeloblasts, erythroblasts, myelocytes, polymorphonuclear leukocytes, lymphocytes, megakaryocytes, and fat tissue) within the human adrenal has been reported frequently (Kovács,[32] Victor,[33] Soós,[34,35] Herzenberg,[36] Paul,[37] Vigi,[38] Jedlička,[39] Arnold,[40,41] Omelskyj,[42] Knabe,[43] Hopf,[44] May,[45] Gierke,[46] Hickel,[47] Mieremet,[48] Collins[10]). Many authors considered this bone marrow to result from some embryonic malformation or from remnants of bone marrow preexisting in the adrenals at the time of birth. The histogenesis of this tissue has always been a mystery and it would hardly be rewarding to discuss the various, highly theoretic possibilities which have been considered. It is noteworthy, however, that here again a surprisingly large number of the patients with such bone marrow tissue in the adrenals suffered from malignant tumors or cardiovascular disease (Collins,[10] Dieckmann[49]). Occasionally myeloid elements developed in so-called accessory cortical tissue near the adrenals (Brian,[50] Herzenberg[36]). This is of some interest since the so-called brown fat tissue or hibernating gland tissue—which occurs around the suprarenals—is especially predisposed to hemopoietic metaplasia as judged by our experiments. In view of the great resemblance between brown fat and

adrenal cortex it is not impossible that the two might have been confused in such cases.

Direct interference with the adrenals, such as may be occasioned by trauma or vascular disturbances, could also enhance hemopoiesis in these glands by stimulating the metaplastic potentialities of reticulo-endothelial elements. Thus, introduction of a celloidin particle into the rabbit adrenal was claimed to cause marked proliferation of the reticulo-endothelial cells with the formation of a granuloma, which contained myelocytes (Ssyssojew,[51] Sserdjukoff[52]). It is not improbable that the so-called myelolipomas (Oberling,[53] Giffen[73]) of the adrenal are not true blastomas but merely represent foci of fat formation with hemopoiesis in these glands. It should also be mentioned in connection with these observations that erythrocytes undergo destruction especially in the zona reticularis of the adrenals (Auld[54]), a region in which the stroma is rich in macrophages (Sabrazès and Husnot[55,56]).

Hemopoiesis in the Renal Pelvis and Retropleural Fat. Several pathologists have reported hemopoiesis in the renal pelvis of man (Schultze,[57] Tanaka,[58] Mieremet,[48] Matsunaga[59]). In some instances these foci merely represented one localization of a more generalized process of myeloid leukemic infiltration, while in other cases they were isolated nodules. Tumor-like retropleural red bone marrow proliferation has also been observed in man (Pick[60]).

It is noteworthy that these proliferations tend to occur in those locations in which the so-called brown fat is seen normally, since in our animal experiments corresponding myelogenous tissue appears to occur by direct metaplasia of this brown fat. These findings are also reminiscent of the bone and bone marrow formation which occasionally occurs in the kidney under the influence of nutritional disturbances. Such heteroplastic bone formation has also been produced in animals, *e.g.*, in the rabbit, after ligature of the renal pedicle (Sacerdotti and Frattin[61]).

In our experiments, as well as in most of the clinical cases mentioned above, hemopoiesis in the adrenals was not accompanied by bone formation; yet ossification, sometimes with the development of typical bone marrow, also has been noted in the human adrenal (Woolley,[62] Newsam,[63] Kruse[64]). It is doubtful whether bone and bone marrow formation in the kidney, renal pelvis, and adrenal tissue are due to related pathogenic agents. We mention them conjointly here only in view of the great structural similarity between the adrenal cortical cell and the brown fat tissue of the renal pelvis and perirenal tissue.

Effect of Hormones on Hemopoiesis and Hemolysis. Several recent

reviews have surveyed the rôle of the endocrine glands in the control of blood formation and blood destruction (Bossak, Gordon, and Charipper,[65] Daughaday, Williams, and Daland[66]). Hence it will not be necessary to review the pertinent literature in detail. Suffice it merely to re-emphasize that many clinical and experimental observations suggest that the anterior pituitary, the adrenal cortical, and testoid hormones tend to stimulate erythropoiesis, while folliculoids may cause severe anemia with a marked decrease in the red cell count, especially in certain predisposed species such as the dog.

It has been possible, furthermore, to produce myeloid infiltrations in various tissues (including the renal pelvis, spleen, and adrenals) in guinea-pigs by injections of extracts of beef liver (Turner and Miller,[67] Meyer and Sawitsky[68]) or of urine of leukemic patients (Miller and Hause,[69] Miller *et al.*[70,71]). In view of our above-mentioned observations with various crude tissue extracts it would be important to ascertain, however, whether these lesions can really be ascribed to specific hemopoietic substances rather than to the admittedly pronounced, local tissue damage caused by these injections.

Following treatment with extremely high doses of folliculoids, especially stilbestrol, hepatic necrosis with icterus can be produced in the mouse. Similar lesions have also been seen in rats although the latter are much more resistant.[72] Simultaneously with the hemolytic icterus, green pigment granules are deposited in the proximal convoluted tubules of the kidney, a process which sometimes is so intense that the color of the organ becomes distinctly green to naked eye inspection. These granules contain free iron as judged by the Prussian-blue reaction. They presumably represent products of increased blood destruction. Recent unpublished observations, made at our Institute, revealed further that anterior pituitary extract augments the hepatotoxic and the icterus-forming action of the folliculoids. Thus, it appears that anterior pituitary preparations can synergize both the erythropoietic effect of testoids and the hemolytic, hepatotoxic actions of folliculoids.

Summary

Experiments on the rat indicate that:

1. Testoid compounds (*e.g.*, methyl testosterone) can cause transformation of adrenal cortical cells into elements exhibiting the characteristic morphologic criteria of ordinary fat cells. Such adipose transformation is not observed in the hypophysectomized animal following treatment with testosterone, but is intensified by simultaneous administration of corticotrophic anterior pituitary extracts.

2. Crude anterior pituitary extracts, rich in corticotrophin, tend to cause marked hemopoiesis in the adrenal cortex. Such an effect has not been produced with purified corticotrophin and appears to depend upon the combined action of the corticotrophic hormone itself and the tissue necrosis occasioned by the crude extract.

3. Under the influence of prolonged, combined treatment with testosterone and crude anterior pituitary preparations, the entire zona reticularis and inner fasciculata is transformed into typical bone marrow containing all of the characteristic elements of hemopoietic tissue as well as fat cells. Some such hemopoietic transformation also occurs in the subcapsular connective tissue layer, but the glomerulosa and outer fasciculata are usually preserved. Thyroxin, which had previously been shown to intensify the corticotrophic action of pituitary preparations, also augmented the hemopoietic effect of the hormone combination used in the present experimental series.

4. The brown fat tissue of the periadrenal region and renal pelvis is likewise transformed into myeloid tissue under the influence of crude anterior pituitary preparations, especially if the latter are administered in combination with methyl testosterone and thyroxin.

5. In the spleen, intense proliferation of lymphoid and myeloid elements, accompanied by the formation of numerous giant megakaryocytes, was induced by an impure anterior pituitary preparation. This effect was also greatly augmented by methyl testosterone and thyroxin.

6. Attention is called to the similarity between these experimentally produced changes and certain spontaneous lesions noted in man; especially in patients suffering from advanced carcinomatosis, atherosclerosis, renal disease, heart disease, or hypertension.

These results are of interest in the light of our earlier experiments, which had shown that hemopoiesis can be induced also in the adrenals of rats by transplantable tumors (if the latter undergo necrosis), by extracts of necrotic tumors, or by necrotic kidney-implants. Apparently chronic intoxication, with products liberated from necrotic tissues, modifies the usual response of the adrenals to non-specific general stress, so that in addition to the usual cortical hypertrophy and hyperplasia, myeloid transformation tends to occur. Our previous work revealed, furthermore, that arteriosclerosis, nephrosclerosis, cardiac lesions, and hypertension can be produced regularly in rats by treatment with crude corticotrophic anterior lobe extracts, similar to those employed in the present experimental series to stimulate myelopoiesis in the suprarenal cortex.

There is considerable evidence that the above-mentioned cardio-

vascular and renal lesions are "diseases of adaptation," due to a derailment of the normal, defensive increase in corticotrophin production, which occurs during adaptation to a variety of systemic stresses (infections and intoxications). Apparently the usual response to exogenous or endogenous excesses of corticotrophin can be so modified by products of necrotic tissues that intense hemopoiesis results.

This type of response will have to be taken into account in the interpretation of hematologic reactions, especially the well known polymorphonuclear leukocytosis, which develops under the influence of non-specific stress during the general adaptation syndrome.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

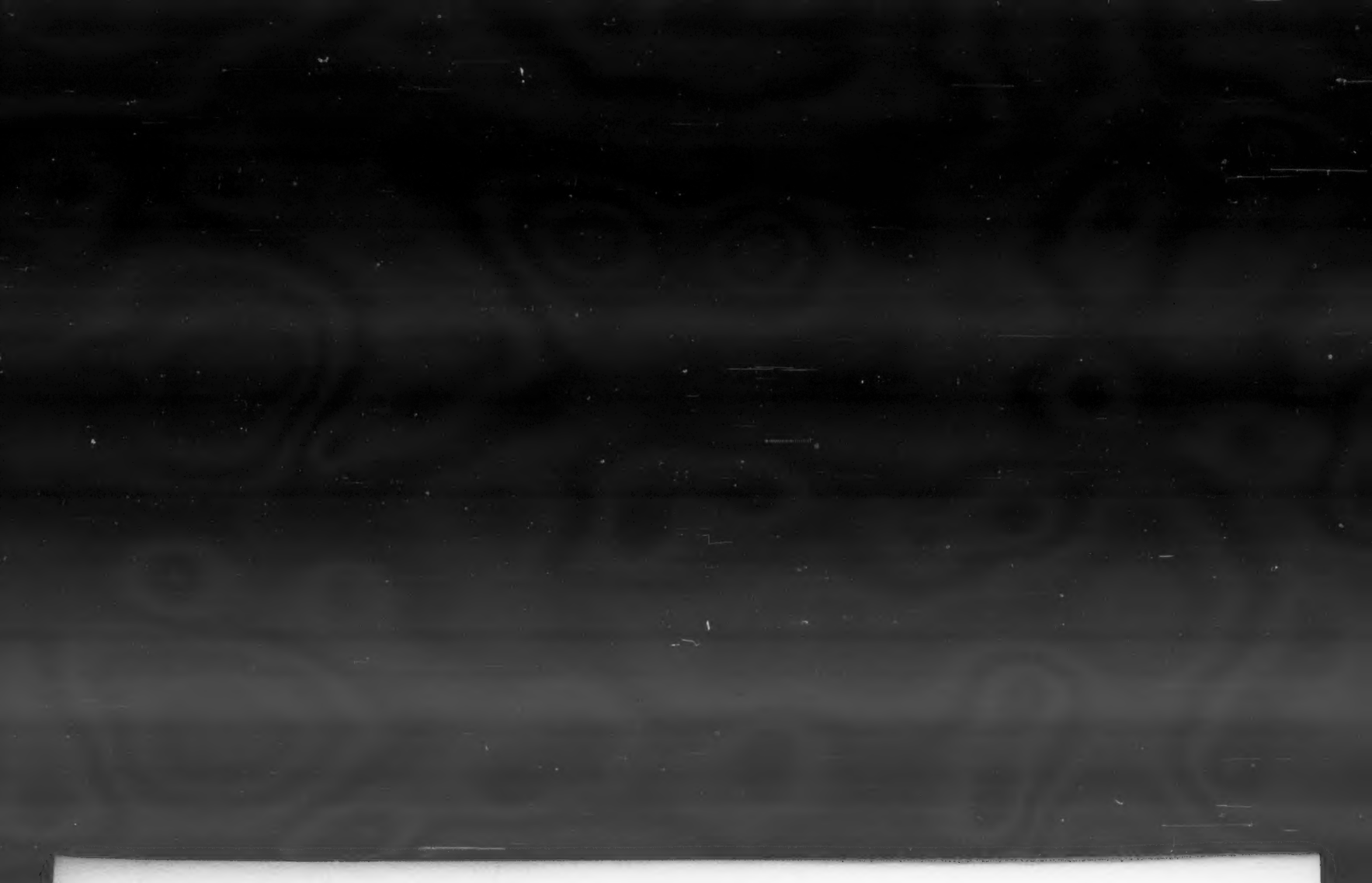
PLATE 36

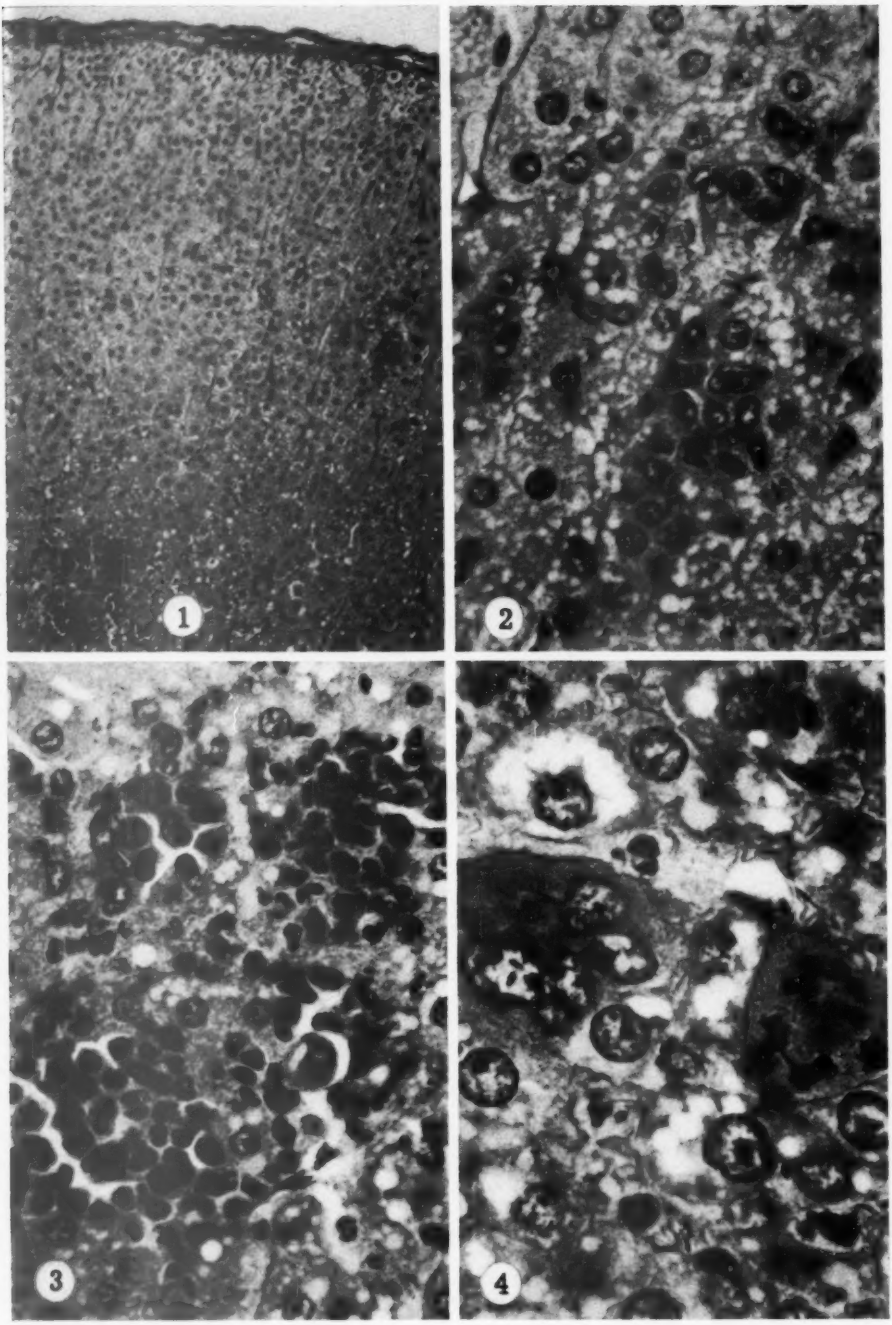
FIG. 1. Adrenal cortex of a rat treated with lyophilized anterior pituitary (LAP) (group V). Two foci of myeloid round cell infiltration are shown in the midst of apparently normal adrenal cortical tissue. × 100.

FIG. 2. The smaller of the two foci shown in Figure 1. There are many transitional stages between the apparently normal adrenocortical cells and small lymphoid elements as well as cells with lobed nuclei. × 450.

FIG. 3. The larger of the foci shown in Figure 1. In this region the myeloid structure of the cortex becomes more evident. Of note are several polymorphonuclear leukocyte-like elements, especially in the right upper corner of the field. × 450.

FIG. 4. Polymorphonuclear giant cell of the megakaryocyte type in the adrenal cortex of a rat treated with LAP, methyl testosterone, and thyroxin (group VII). On the right, a similar cell showing multipolar mitosis. × 1000.





Selye and Stone

Transformation of Adrenal into Myeloid Tissue

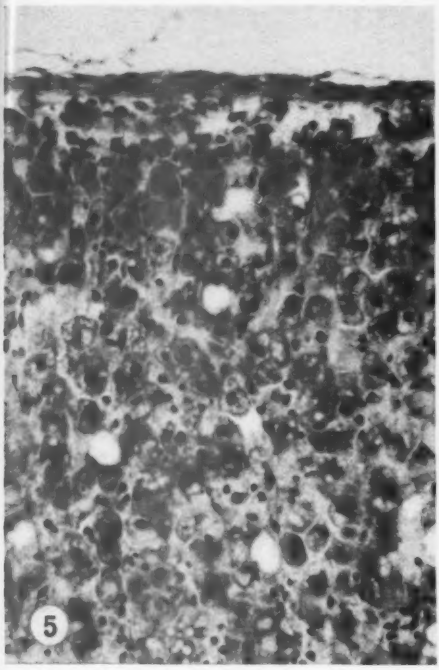
PLATE 37

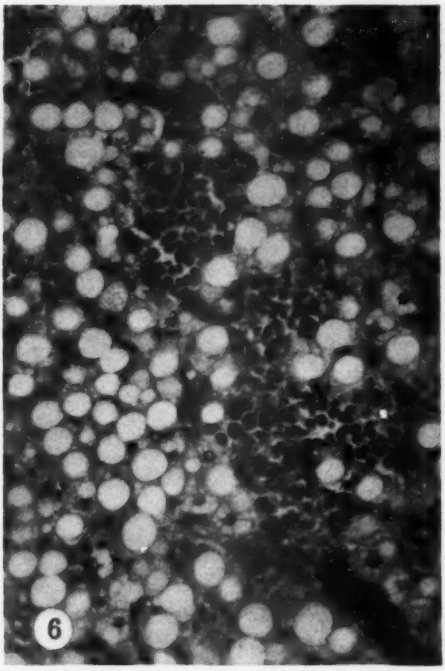
FIG. 5. Fat cell formation in the inner fasciculata of another rat from group VII. There is marked fat cell formation, but myeloid elements are not very prominent in this field. Only scattered round cell accumulations are detectable in the stroma. Adrenal cortex is still recognizable. × 200.

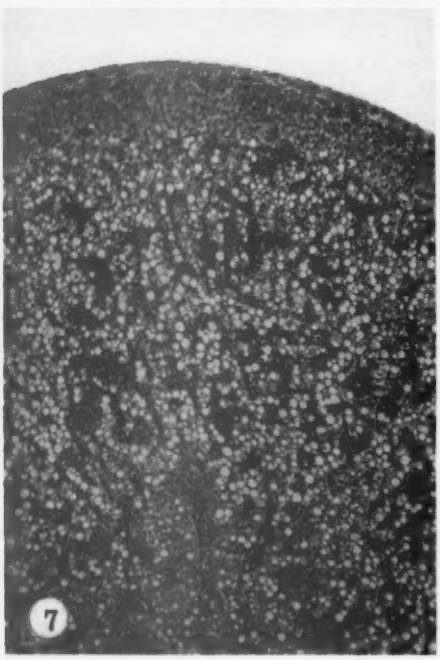
FIG. 6. Myeloid tissue from the inner fasciculata region of another rat of group VII. Of note are numerous fat-cell-like elements in the stroma, and myeloid foci. The tissue resembles normal bone marrow; adrenal cortical cells are no longer recognizable. × 200.

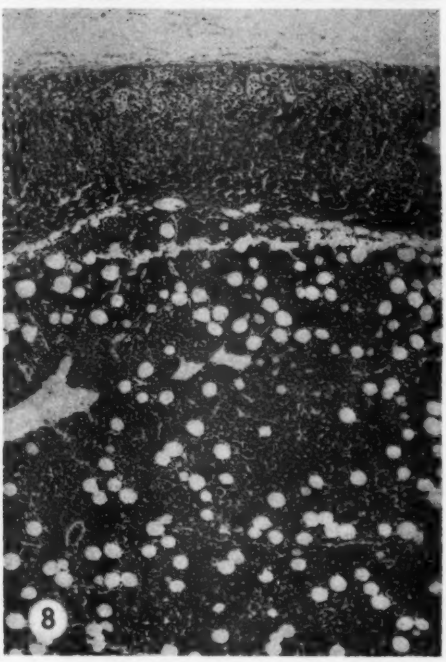
FIG. 7. General aspect of the adrenal cortex of a rat from group VII under low magnification. Of note are the essentially intact glomerulosa and outermost part of the fasciculata while the entire inner fasciculata and reticularis are no longer recognizable as adrenal tissue; they assumed the typical appearance of bone marrow tissue. × 45.

FIG. 8. Myeloid tissue spontaneously occurring in the human adrenal (after Collins[10]), showing a great resemblance to the experimentally produced myeloid metaplasia seen in Figure 7. Here again the glomerulosa and outer fasciculata are preserved, while the inner layers are replaced by typical bone marrow tissue. × 45. (Reproduced by permission from *The American Journal of Pathology*, 1932, **8**, 97–105.)









Selye and Stone

Transformation of Adrenal into Myeloid Tissue

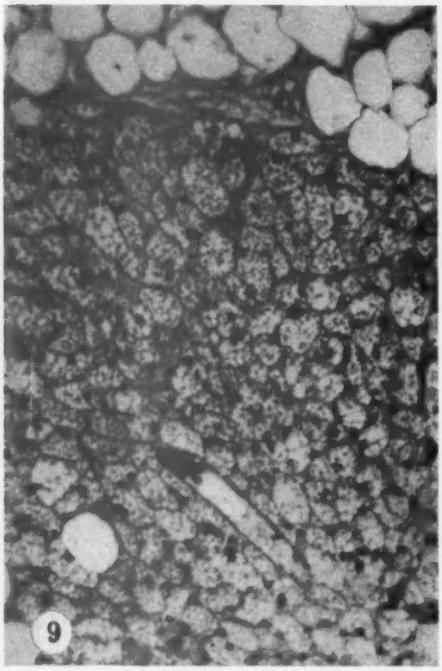
PLATE 38

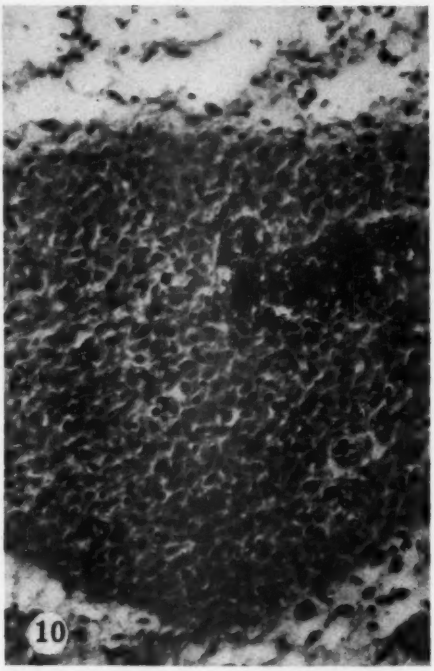
FIG. 9. Brown fat tissue embedded in the fat surrounding the renal pelvis of a normal rat. Of note is the resemblance of these cells to those of lipid-storing, adrenal cortical cells as well as the marked difference between the brown fat and ordinary fat cells in this field. × 200.

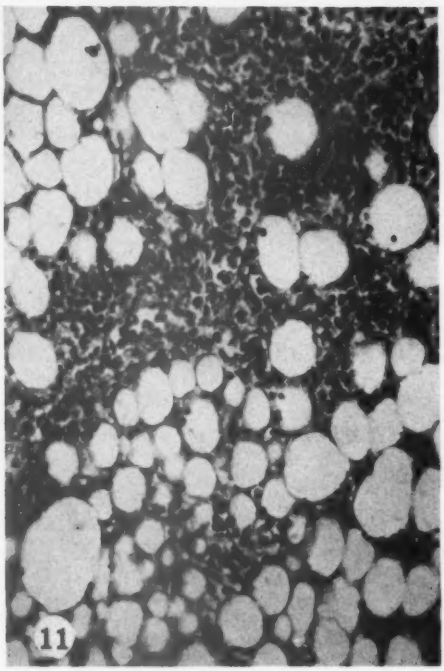
FIG. 10. Brown fat tissue from the renal pelvis region of a rat of group VII, showing loss of lipid granules and decrease in the size of the brown fat cells. In this field the resemblance to degranulated adrenal cortical tissue is quite striking. × 200.

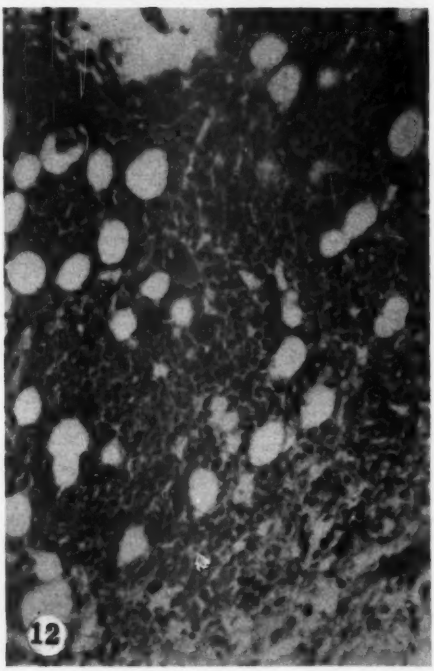
FIG. 11. Brown fat tissue from the region of the renal pelvis of a rat of group V. There is beginning myeloid transformation of the brown fat tissue elements. × 200.

FIG. 12. Brown fat tissue from the renal pelvis of another rat from group V. Here the myeloid transformation has progressed farther and several megakaryocyte-like cells have appeared. × 200.









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Transformation of Adrenal into Myeloid Tissue

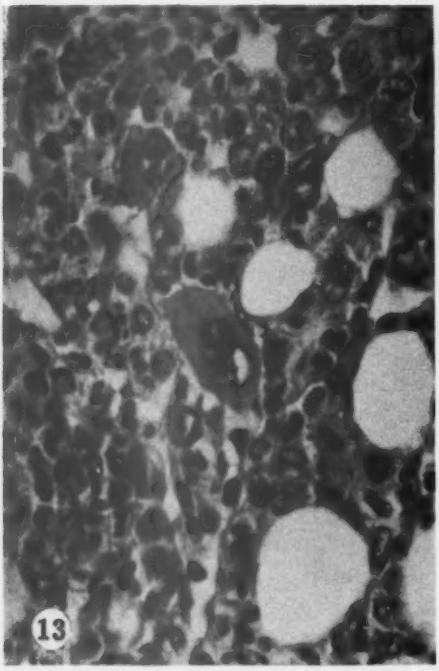
PLATE 39

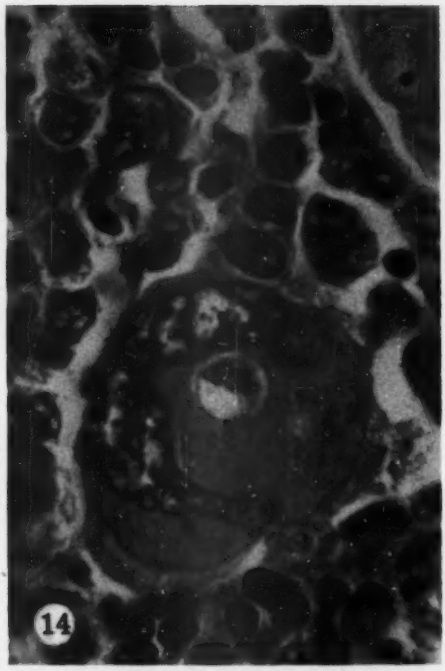
FIG. 13. Central region from Figure 12. Of note are the megakaryocyte-like giant cell and the numerous neutrophil and eosinophil leukocytes, myelocytes, erythroblasts, and other myeloid elements. × 450.

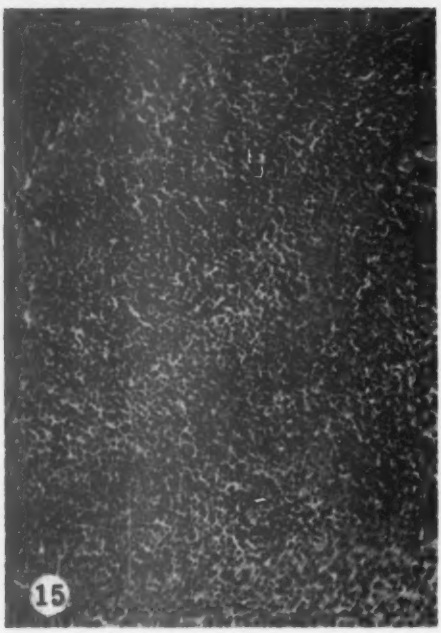
FIG. 14. Region from the brown fat tissue of a rat from group VII. The typical megakaryocyte-like cell encloses a degenerating polymorphonuclear leukocyte and emits several pseudopodium-like processes from the cell surface. The surrounding cells are also typical of hemopoietic tissue. × 1000.

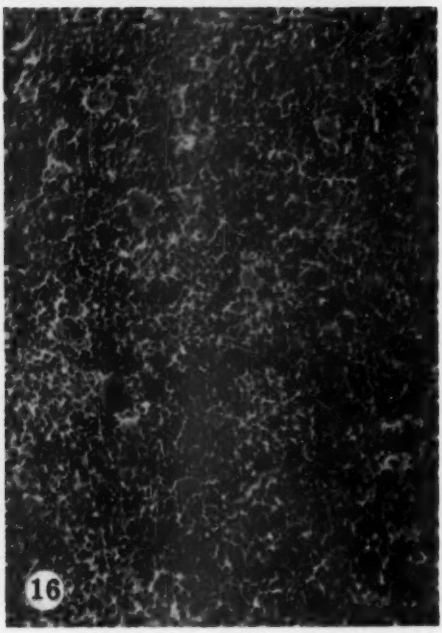
FIG. 15. Spleen of a normal control rat of group I. × 100.

FIG. 16. Spleen of a rat from group VII. There is marked proliferation of the hemopoietic elements with formation of numerous very large megakaryocytes. × 100.









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Transformation of Adrenal into Myeloid Tissue

THE EFFECT OF RENAL ISCHEMIA ON THE PRODUCTION OF EXPERIMENTAL NEPHROSIS IN JAUNDICED RABBITS*

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The purpose of the experiments described in this paper was to test the assumption that previous renal damage might be a factor in the development of bile nephrosis. Evidence has been produced by Yuile, Gold, and Hinds[1] that such a factor is important in the development of nephrosis following the injection of hemoglobin into rabbits. They injured the kidneys moderately, either by obstructing the renal artery for a short period or by administering a small dose of sodium tartrate. When hemoglobin was injected intravenously after these procedures, it was precipitated in the tubules of the kidney. In control animals with uninjured kidneys no such precipitation of hemoglobin occurred. These experiments suggest that a similar mechanism may be at work in bile nephrosis and that those patients who develop renal disease when they are jaundiced do so because they have previously damaged kidneys. In order to test this supposition, experiments were performed in which one kidney was damaged by a short period of renal ischemia, accomplished by occluding the right renal artery and vein, after which the common bile duct was divided in order to produce jaundice. The kidneys were then examined after various intervals.

EXPERIMENTS

Male and female rabbits of mixed laboratory stock and weighing 1.5 to 3.4 kg. were used. They were fed purina chow pellets and allowed as much water as they would drink. Vegetable scraps were added to the diet from time to time.

The surgical procedures were performed aseptically under ether anesthesia, and care was taken to cause as little trauma as possible. A single vertical incision in the right upper quadrant of the abdomen, afterwards closed with a single row of interrupted silk sutures, was used in all of the operations. The common bile duct was doubly ligated and divided between the ligatures. The main renal artery and vein were raised on an aneurysm needle and occluded for 12 to 20 minutes. The animals recovered from the operation and the wounds healed promptly without infection.

At the conclusion of the experiments the rabbits were killed with ether and autopsies were done immediately. Pieces of liver and of both

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kidneys were placed in 10 per cent neutral formalin and Zenker-formol and fixed for 24 hours. The blocks were embedded in paraffin, and sections were cut and stained with hematoxylin and eosin and by Masson's trichrome and McManus'[2] periodic acid methods. Hemoglobin was demonstrated by Lepehne's benzidine technic.[3] Frozen sections of formalin-fixed tissue were stained with sudan IV. In order to study the casts in the renal tubules, frozen sections of fresh unfixed as well as formalin-fixed kidney were examined.

The animals were divided into the following groups. Group 1 consisted of 12 rabbits in which the common bile duct was divided and the right renal artery and vein were occluded. In 9 animals the renal vessels were occluded for 12 minutes and in 3 for 20 minutes. Autopsies were performed 2, 4, 6, 7, 9, 12, 14, 16, 22, and 33 days after the operation.

Group 2 consisted of 4 rabbits in which only the common bile duct was divided and nothing was done to the renal vessels in order to study the effect of jaundice in the absence of renal ischemia. These animals were killed 3, 8, 11, and 14 days after the operation.

Group 3 consisted of 7 rabbits in which only the right renal artery and vein were occluded. In 5 animals they were occluded for 12 minutes and in 2 for 20 minutes. They were killed 4, 7, 8, 14, 17, and 18 days after the operation. This group was the control for the effect of renal ischemia in the absence of jaundice.

Group 4 consisted of 2 rabbits in which the common bile duct and right renal artery and vein were exposed but not otherwise manipulated. These experiments were done to test the effect of the operation alone. One animal was killed after 7 days and one after 18 days.

Group 5 consisted of 4 rabbits which were kept under the same laboratory conditions as the other animals but were not subjected to any operative procedure. They were killed at intervals and sections of all organs were examined.

Results

Group 1. Lesions Observed in Rabbits with both Jaundice and Renal Ischemia

The rabbits in group 1 showed lesions in both kidneys consisting of pigmented casts within dilated tubules, degeneration, necrosis, and flattening of the tubular epithelium. Clinically, all rabbits which lived longer than 3 days were jaundiced, but none showed suppression of urine, edema, azotemia, or other evidences of renal damage. The urine of all rabbits was kept neutral or alkaline because this is the natural

reaction for healthy rabbits and it was desired to introduce as few abnormal conditions as possible. The pertinent clinical and pathologic data for these animals is given in Table I.

Except for generalized icterus and renal damage, the only other lesions were in the liver where there was biliary obstruction, the severity of which depended upon how long the animal lived after the common bile duct was divided.

TABLE I

The Influence of Renal Ischemia on the Production of Nephrosis in Jaundiced Rabbits

Rabbit no.	Reaction of urine	Right renal vessels occluded	Effect on urine output	Survival	Microscopic examination of kidneys		
					Pigmented casts	Dilated tubules	Necrosis of tubules
		minutes		*days*			
2204	Alkaline	12	None	6 K*	+++	+++	o
2205	Alkaline	12	None	2 D†	+++	+++	+++
2206	Alkaline	12	None	12 D	+++	+++	o
2211	Alkaline	12	None	14 K	o	++	o
2213	Alkaline	12	None	33 K	+++	+++	o
2215	Alkaline	12	None	9 K	+	+	o
2216	Alkaline	12	None	9 D	o	o	o
2224	Neutral	12	None	16 K	+	o	o
2252	Acid	12	None	4 D	o	+	o
2222	Alkaline	20	None	7 D	++	++	o
2228	Alkaline	20	None	22 K	o	o	++
2230	Alkaline	20	None	2 D	+	+	o

* Killed with ether vapor.
† Died.

Grossly, the kidneys were diffusely stained with bile. They were of normal size and the capsules stripped easily leaving smooth, dark red surfaces. The pattern of the cut surfaces was well preserved and distinct. The pelves and ureters were normal.

Microscopically the glomeruli were normal except for an occasional one which contained a small amount of faintly eosinophilic material, probably precipitated protein, in the space of Bowman. The blood vessels and pelves were normal.

The most important alterations were discovered in the tubules of the cortex. Not all tubules were attacked and it was estimated that in the most severely damaged kidneys about 25 per cent were involved, the remainder appearing histologically normal. It was difficult to localize the lesions to any specific portion of the renal tubules, for even in the diseased rabbit kidney the identification of the proximal and distal segments was uncertain. It was our impression that the distal portion of the nephron was predominantly affected, but changes also were observed in the proximal portion. Despite the fact that only the right renal artery and vein were occluded, identical histologic lesions were present in both kidneys. The severity of the lesions varied in different

animals, but was always equal in the two kidneys of the same animal. Thus 4 rabbits showed marked histologic alterations in the kidneys, 3 moderate, and 4 slight. Only one rabbit had kidneys which were entirely normal histologically.

Pigment Casts. Perhaps the most striking lesion was the presence of numerous orange-red casts in the dilated tubules (Figs. 2 and 3). Although these pigmented casts were located principally in the distal segments, they also were present in the proximal tubules and collecting ducts. Most of them were granular and many bore a striking resemblance to red blood cells (Figs. 1 and 3). Others had the appearance of short lengths of copper wire (Fig. 2). A few were smooth. These casts gave a positive benzidine reaction with the Lepehne technic, indicating that they contained hemoglobin (Figs. 1 and 4). They gave negative histochemical reactions for iron and fat and with periodic acid. Histochemical tests for bile salts proved unreliable.

Pigment, which had a similar appearance and gave identical histochemical reactions, was present also in some of the intact epithelial cells as well as in many of the detached cells within the lumen (Figs. 3 and 4).

Besides the heme casts, there were also bile-stained casts. These were best seen in unstained frozen sections of fresh tissue and were either granular or hyaline. In fixed and stained sections they were inconspicuous, the pigment probably having been washed away during the preparation or obscured by the dyes.

Other Casts. Hyaline casts which stained intensely with eosin and green or red with Masson's trichrome stain were common, and there were a small number of casts of coagulated protein material which were pale and finely granular. Such casts were found chiefly in the distal and collecting tubules, although granular casts were found also in small numbers in the proximal tubules.

Dilatation of Tubules. The affected tubules were dilated, lined by flattened epithelial cells (Figs. 1 to 4), and usually contained heme casts. In most cases it was the distal segment of the tubules which was dilated.

Necrosis of Tubular Epithelium. Necrosis of tubular epithelium was present in only 2 cases. It was not very extensive and characteristically involved only short segments (Fig. 5). Exfoliated necrotic cells lay free in the lumen or were mixed with the hemoglobin casts (Fig. 3).

Flat Tubular Epithelium. Many of the tubules, particularly the dilated ones, were lined by thin, flat epithelial cells with scanty cytoplasm and small, centrally placed nuclei (Figs. 1 to 4). It may be

that these cells were regenerating epithelium, but this is by no means certain for there were no mitotic figures and usually no clear evidences of necrosis. If these were regenerating epithelium, regeneration must have occurred very early for they were found as soon as 4 days after operation.

Thickening of the Basement Membrane of Tubules. A peculiar irregular thickening of the basement membrane of a few tubules was disclosed by the periodic acid technic, the affected membranes staining intensely and uniformly with the Feulgen reagent (Fig. 6). McManus[2] believed that this is an indication that the tubule is undergoing atrophy. This lesion was present only in one rabbit which had survived operation for 33 days, so that it is probably a relatively late change, and may indicate that permanent damage may result.

Pigment in the Tubular Epithelium. In addition to the granular hemoglobinogenous pigment, which has been described before, there was, in many cases, a deposit of granular yellowish green pigment in the epithelium lining the proximal convoluted tubules. This was thought to be bile pigment because of its green color and the fact that it was more abundant in frozen sections of unfixed tissue than in the stained sections cut from paraffin blocks. However, we were unable to establish the nature of this pigment with certainty.

Degenerative Changes in the Tubular Epithelium. Degeneration of all parts of the tubule was present in the majority of the animals. The principal changes were cloudy swelling, vacuolization, and hyaline droplet formation. Fatty degeneration did not occur.

Groups 2, 3, 4, and 5

Only one of the 17 rabbits in the control groups 2, 3, 4, and 5 showed renal lesions. This was one of the 4 animals in group 2, in which the common bile duct had been ligated and divided. This animal showed an occasional dilated tubule containing a heme cast. Occlusion of the renal artery and vein in the 7 animals in group 3 for periods of 12 and 20 minutes caused no demonstrable lesions 4 to 18 days after operation.

Discussion

Before going on to a discussion of this experimentally produced renal disease, it will be helpful to review briefly the available information about bile nephrosis in humans in order to have a basis for comparison of the two lesions.

Human kidneys in a typical case of uncomplicated bile nephrosis are likely to be large and diffusely stained with bile. The capsule is

tense and strips easily, leaving a smooth surface. The organ cuts without increased resistance and the cut surfaces are wet, bulge, and have prominent striae. The pelves are normal. Microscopically, the majority of glomeruli are normal and only an occasional one shows slight focal proliferation of endothelium. But, profound changes may occur in the tubules. This fact is widely recognized, but there is a difference of opinion about the location and the nature of these changes. Most writers[4-6] state that the essential changes are found in the first portion of the tubules, especially the proximal convoluted tubules, and that they are similar to those produced by mercuric chloride. Thus the epithelial cells lining the proximal tubules are said to show cloudy swelling, vacuoles, hyaline droplets or other degenerative changes, and, in severe cases, necrosis. Regeneration of flattened epithelium frequently goes hand in hand with the necrosis. The lumina may be dilated and filled with precipitated protein, exfoliated epithelial cells, and casts, which are frequently bile-stained. Bile pigment is deposited also in the cells lining the proximal portion of the tubules. Exudate is not present, either in the tubules or in the interstitial tissues. The lower portion of the nephron and the collecting tubules are spared except for casts and débris which have presumably been washed down from above. The descriptions of these authors indicate that the lesions of bile nephrosis are not the same as those of the lower nephron syndrome (crush kidney, transfusion kidney), and Lucké[7] does not include bile pigment in the list of agents which may produce nephrosis of the lower nephron.

However, the papers of Ayer[8] and Ayer and Gould[9] described different changes. These authors[9] examined the kidneys of infants who died in deep jaundice due to congenital atresia of the bile ducts. "All the essential features of the renal changes of the post-transfusion reaction could be found in the kidneys of jaundiced infants with congenital atresia of the bile ducts. In the kidneys of these infants edema and leukocytic infiltration of the interstitial tissue took place only about the distal convoluted tubules and collecting ducts. Necrosis of epithelial cells occurred only in these two segments. The simplest change in these segments was the appearance of bright red material (often tinged with brown) in epithelial cells." Their descriptions are illustrated by excellent photographs of the microscopic lesions.

Thus there is disagreement about the morphologic features of bile nephrosis and at present we do not know whether the lesions are

similar to those of mercuric chloride poisoning, or to those of the lower nephron syndrome. Perhaps in some cases the kidneys may show a combination of both types. A careful study of the kidneys from jaundiced patients should supply this information.

Because of the incompleteness of our knowledge of the lesions of bile nephrosis, it is difficult to interpret the experimental findings described in this paper. Definite changes were present in the tubules of both kidneys within a few days after division of the common bile duct and obstruction of the right renal artery and vein for a period of 12 to 20 minutes. No changes were discovered in rabbits in which only the right renal artery and vein were obstructed. Nor did rabbits in which the common bile duct and right renal pedicle were isolated but not otherwise manipulated show changes in the kidneys. One of the 4 control rabbits in which the common bile duct was ligated did show renal lesions similar to those in the experimental animals, but they were of only slight degree.

The finding of lesions of approximately equal intensity in both kidneys, although only the right one had had its blood supply impaired, was unexpected and remains unexplained. A similar experience was reported by Yuile, Gold, and Hinds in experimental hemoglobinuric nephrosis.[1] The absence of comparable lesions in the control animals indicates that they did not result from trauma associated with the operations. Perhaps they were due to a reflex nervous mechanism similar to that described by Trueta and his associates.[10] In this connection it should be mentioned that no effort was made to spare the nerves in the renal pedicle and they may have been stimulated unwittingly when the artery and vein were obstructed with the aneurysm needle.

The paucity of clinical evidences of renal disease in the animals was probably due to the fact that the lesions were focal and affected only a relatively small number of tubules. Some animals even escaped demonstrable damage entirely. It should be remembered in this connection that the rabbits were allowed as much water as they would drink, and it may be that if the water intake had been restricted, more extensive morphologic lesions accompanied by clinical evidences of renal disease would have resulted. The recent work of Lalich[11] on hemoglobinuric nephrosis suggests this possibility.

The presence in the damaged tubules of reddish brown casts, which gave the staining reactions of hemoglobin, and of granules with like properties within the tubular epithelial cells, was unexpected because

such findings usually are associated with the lower nephron syndrome. We had expected to find large numbers of bile-stained casts, but in reality these were less numerous than hemoglobin-containing casts. The presence of these hemoglobin casts is unexplained by these experiments. It may be that in some unperceived way a true lower nephron nephrosis was produced, for instance by the stimulation of the nerves in the renal pedicle or elsewhere, as has been mentioned above. However, the absence of similar changes in the control animals does not support this idea.

Although the changes which occurred in the kidneys of these rabbits resemble in some respects those which are seen in the lower nephron syndrome, there were the following differences: (1) There was no exudate in the tubules, (2) the interstitial tissues were not affected, (3) there was no rupture of tubules into adjacent veins, (4) the location of the lesions was probably not typical, for they seemed to occur in the proximal as well as in the distal segments of the tubules, and (5) renal failure did not occur.

Summary

Under the conditions which prevailed in these experiments, obstruction of the right main renal artery and vein for short periods of time in rabbits which had been made icteric by division of the common bile duct was followed within 4 days by the appearance of lesions in both kidneys. These changes were observed for as long as 33 days. They consisted of dilatation, degeneration, and necrosis of the lining epithelium of the tubules of the cortex and the presence of numerous reddish brown granular casts which gave the histochemical reactions of hemoglobin. Similar pigment was present also in the cytoplasm of some of the epithelial cells lining the tubules. Many of the dilated tubules were lined by flattened epithelium. These lesions were not accompanied by clinical evidences of renal disease, were not of equal intensity in all animals, and in an occasional animal were absent.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

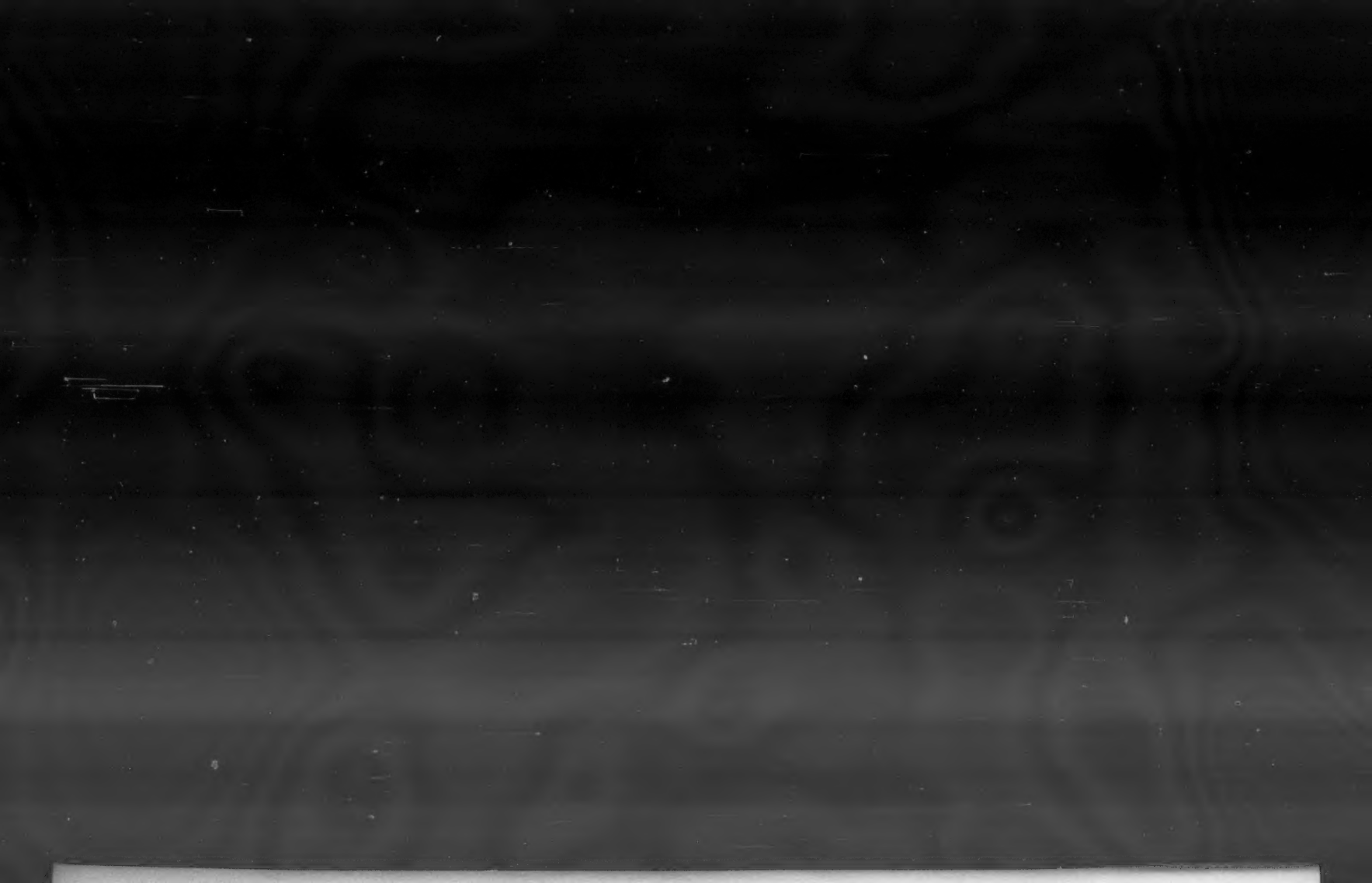
PLATE 40

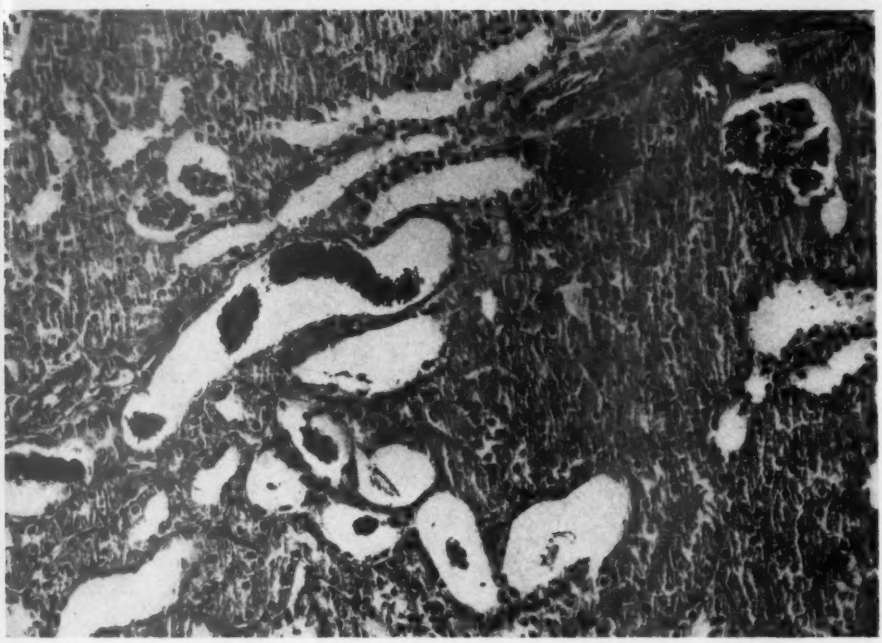
FIG. 1. Rabbit 2206. Outer portion of renal cortex showing granular hemoglobin casts in dilated tubules lined by flat epithelium. Lepehne stain. × 217.

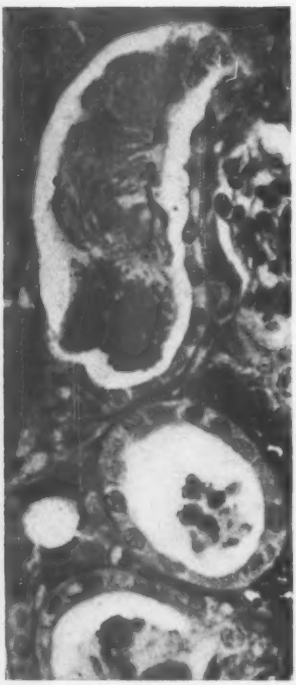
FIG. 2. Rabbit 2204. Hemoglobin casts showing granular and copper wire forms. Hematoxylin and eosin stain. × 600.

FIG. 3. Rabbit 2204. Granular hemoglobin casts and exfoliated epithelial cells. Hematoxylin and eosin stain. × 375.

FIG. 4. Rabbit 2206. Hemoglobin casts and exfoliated epithelium containing hemoglobin in dilated tubule lined by flat epithelium. Hemoglobin deposits are present also in some of the tubular epithelium. Lepehne stain. × 575.







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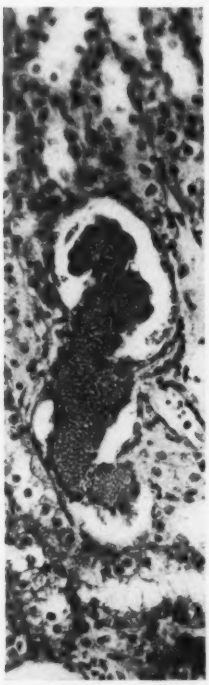

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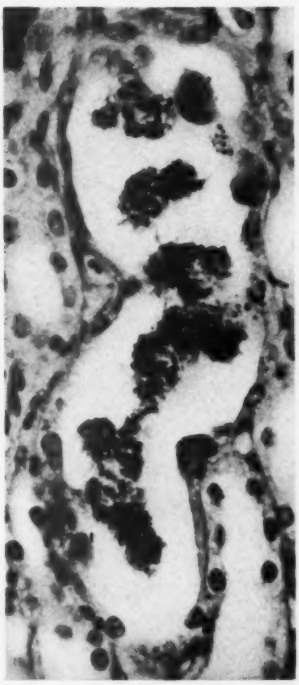


4

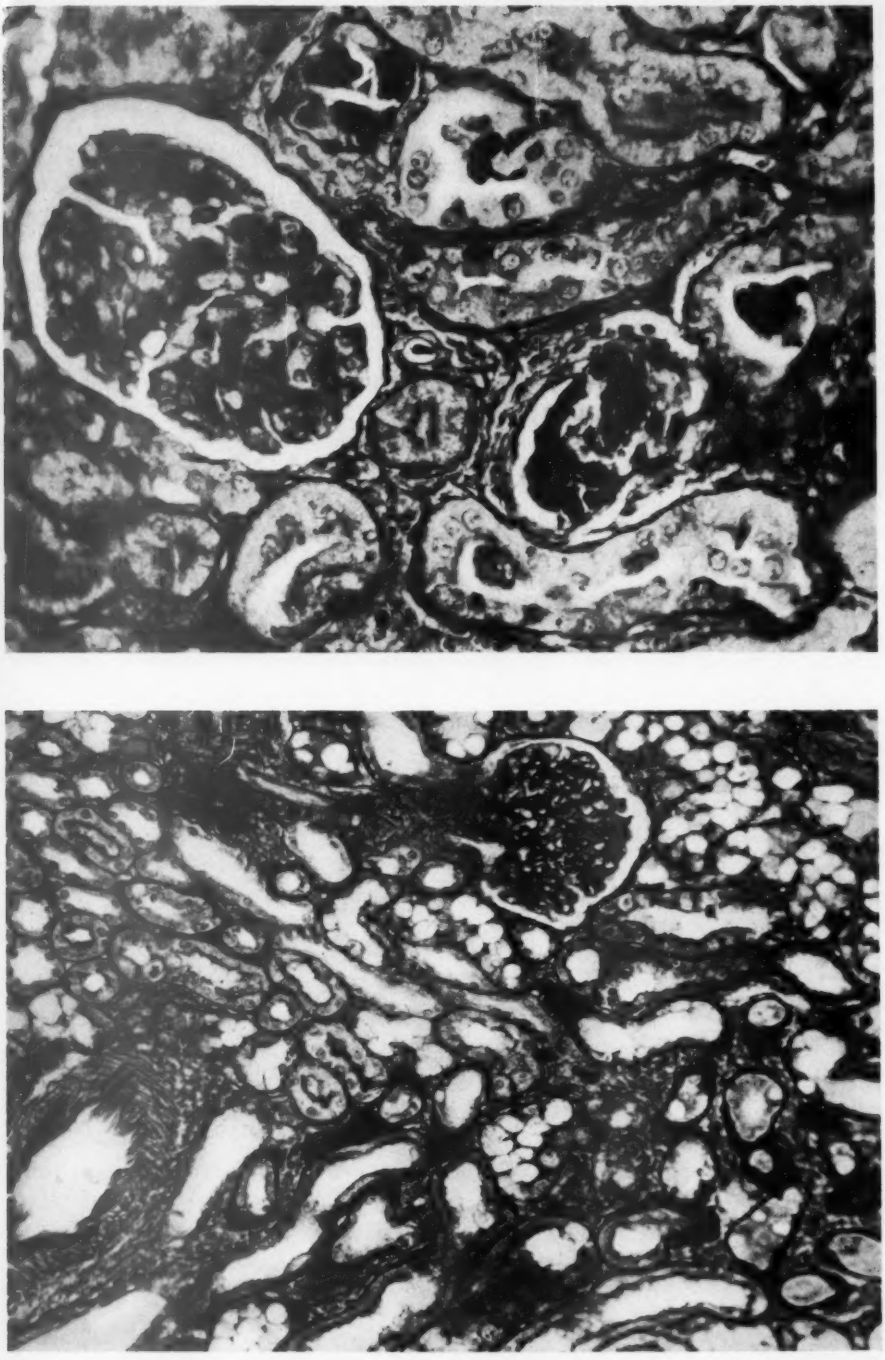
Wartman, Rusterholz, and Tucker

Experimental Nephrosis in Jaundiced Rabbits

PLATE 41

FIG. 5. Rabbit 2205. Necrosis of tubular epithelium. Periodic acid stain. × 600.

FIG. 6. Rabbit 2213. Thickening of the tubular basement membrane. Killed 33 days after ligation of common bile duct and occlusion of the renal artery and vein. Periodic acid stain. × 300.



Wartman, Rusterholz, and Tucker Experimental Nephrosis in Jaundiced Rabbits

CENTRILOBULAR HEPATIC NECROSIS FOLLOWING CARDIAC INFARCTION*

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This is a report on the incidence and morphologic features of centrilobular necrosis of the liver following myocardial infarction.

Centrilobular necrosis is a common finding at autopsy. It has been described in such conditions as trauma, postoperative shock, the crush syndrome, burns, infections, blackwater fever, anaphylaxis, high-altitude flying, and experimental shock.[1] It probably has been overlooked following myocardial infarction because it may be obscured by, and confused with, the changes of congestive heart failure.

The present study is based on the autopsy records of all cases of myocardial infarction of less than 6 weeks' duration in the files of the Banting Institute in the 5-year period ending June, 1948. There were 61 cases in 1793 autopsies. Fifty autopsies from the same period without myocardial infarction, but in which congestive heart failure was diagnosed either clinically or at autopsy, were reviewed as controls. Nine of the cases of infarction had typical centrilobular hepatic necrosis, whereas this was found in only one of the control cases.

Application of the χ^2 test to these results shows that they would occur less than once in twenty times by chance and they are therefore considered significant.[2]

Histologic Changes

The earliest lesions seen consisted of dissociation of the hepatic cells in the centrilobular areas with pyknosis of their nuclei. There was increased eosinophilia of the cytoplasm with little or no shrinkage or swelling. This gave the necrotic areas in the liver a loss of structure and change in color so that they were easily picked out with the low power of the microscope (Figs. 1 and 2). This change occurred less than 24 hours after myocardial infarction, which is in agreement with its time of development in the crush syndrome.[3] The lesions usually surrounded the central veins, but sometimes extended to become almost confluent. In other cases they involved only segments beside central veins, and some lesions appeared mid-zonal, although this may have been due to their being cut in a plane that missed the adjacent vein. In some cases hemorrhage had occurred in the necrotic areas, making them difficult to distinguish from the changes of passive congestion. In the next 5 days the lesions were rapidly surrounded

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and infiltrated by polymorphonuclear leukocytes. The nuclei of the hepatic cells disappeared, their cytoplasm lost its outlines and began to disintegrate (Fig. 3). There were not sufficient cases in the series to time the progress of the lesions accurately.

In a few of the sections there was patchy lipidic vacuolation of the liver cells in the necrotic areas. This vacuolation had probably been present before the infarction. These cells were not so rapidly absorbed, for their outline containing the fat droplet persisted in the otherwise empty stroma (Fig. 4).

When the dead liver cells were absorbed, there remained sinusoids filled with blood, and the lesions then became indistinguishable from those of passive congestion.

The reticulin fibers in the areas of necrosis were not destroyed, and apparently the liver cells may be quickly regenerated to form normal liver. Examination of these cases and many others in which death occurred longer than 6 weeks after the myocardial infarction showed no evidence of permanent lesions that could have resulted from centrilobular necrosis. In the presence of heart failure, however, regeneration of liver tissue did not occur.

In the controls, the well known congestion and centrilobular atrophy and degeneration of chronic venous congestion were frequently found. In the most advanced stage of this, there might be a few necrotic liver cells and some associated polymorphonuclear infiltration, but these inflammatory cells were never focal. Perhaps necrosis of liver cells in such cases is due to the high degree of circulatory failure that may exist for a few hours before death. It can be distinguished by the gradual atrophy and degeneration of the liver cell columns as they approach the central vein. It is most marked in cases of cor pulmonale. The hepatic lesions in chronic passive congestion have been well described by Lambert and Allison.[4] One of their five types of lesions was centrilobular necrosis identical with that described here as being due to myocardial infarction and not to congestive failure. It is significant that the two examples they give for this group are both obvious cases of recent myocardial infarction, their case 9 being proved such by autopsy. It is a common error to attribute these necrotic lesions to congestive failure rather than to myocardial infarction and the associated shock. In the one control case with typical centrilobular necrosis of the liver there was adequate explanation in the lungs for the hepatic lesions on the basis of anoxia. The left lung consisted of fibrous tissue and bronchiectatic cavities and sank in

water. The right weighed 1330 gm. and the functioning parenchyma was largely emphysematous or replaced by tuberculous consolidation and cavitation. The same hepatic lesion has been seen in lobar pneumonia when large parts of the lungs were involved and there was no cardiac failure.

Clinical Data

Data relating to the 9 cases with necrosis of the liver are summarized as follows:

Case no.	Age and sex	Age of infarct at death; clinical and microscopic	Weight of heart	Blood pressure before infarction	Blood pressure at time of infarction
	yrs.		*gm.*	*mm. of Hg*	*mm. of Hg*
1	M 62	7 hours	330	190/105	80/50
2	M 61	19 hours	400	170/110	Not obtainable
3	F 44	About 24 hours	725	200/120	80/60
4	M 68	2 days	457	150/90	170/105
5	M 52	6 days	860	?	74/55
6	F 41	About 14 days	520	210/140	?
7	M 73	18 days	485	?	"Low"
8	M 68	3 weeks	350	250/130	130/90
9	M 45	4 weeks	415	185/100	?

In 6 of the cases with typical hepatic necrosis the patients died within 2 weeks of the myocardial infarction, and it is in this interval that necrosis should be most common. The cases in which death occurred 18 days and 4 weeks after the myocardial infarction had embolic complications, and the patient dying 3 weeks after the infarction continued to have pain in this interval. The lesions in these cases were characteristic, and it is probable that they were due to some circulatory disturbance occurring after the original infarct.

Clinically, shock was commented upon in 5 of the cases with centrilobular hepatic necrosis. This was more frequent than in the cases of myocardial infarction without necrosis of the liver. The other factor that appeared to be significant was hypertension. This was marked in 3 cases, present in 3, and suggested in 2 others by the cardiac hypertrophy in the absence of valvular disease. It would seem reasonable that the decrease in hepatic arterial blood supply would be greater when the fall in blood pressure was greater, and the peripheral resistance of the circulatory system increased.

Discussion

The cause of the liver necrosis is probably acute anoxia due to shock occurring at the time of the myocardial infarction. The differ-

ences between this lesion and the changes in passive congestion have been pointed out in detail. The development of necrosis is not dependent on congestive heart failure, for it occurred in cases in which there was no evidence of heart failure either clinically or at autopsy. Other explanations for these lesions in shock have been offered. A conditioning nutritional factor has been suggested by Himsworth.[5] He has suggested also that swelling of the peripheral cells of the lobule may shut off the circulation to the centrilobular cells in carbon tetrachloride poisoning in animals,[6] but in shock in general and in my cases in particular the peripheral cells in the lobule appear normal at all stages and there is no clinical or pathologic evidence of portal obstruction at any time. Maegraith's theory[7] that similar lesions in blackwater fever are due to valves in the hepatic veins causing back pressure is based chiefly on evidence from dogs, in which such valves are prominent and the liver is one of the organs most affected by shock. Centrilobular necrosis does not occur in experimental obstruction of the inferior vena cava,[8,9] nor in spontaneous obstruction of the hepatic veins in man.[10] The theory that the liability to damage of the center of the lobule depends on a progressive fall in oxygen tension in the blood traversing the sinusoids has yet to be disproved, and would explain the distribution of necrosis in conditions of circulatory failure and collapse.

Summary

Focal necrosis in the liver following cardiac infarction presents characteristic morphologic features. It is to be differentiated from the changes of passive congestion.

It was found in 9 of 61 consecutive autopsies with recent myocardial infarction.

The lesions are recognizable in the first 2 weeks following cardiac infarction, and disappear within 1 month leaving no permanent hepatic changes.

They are the same as the hepatic lesions found in shock from any cause, and are believed to be due to acute anoxia.

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[*Illustrations follow*]

DESCRIPTION OF PLATE

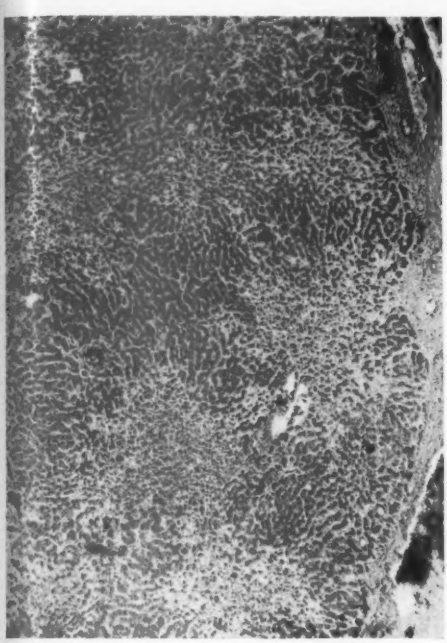
PLATE 42

FIG. 1. Early centrilobular necrosis. × 40.

FIG. 2. Higher magnification of one of the lesions in Figure 1 showing cell dissociation and necrosis. × 300.

FIG. 3. A later stage of necrosis with infiltration of polymorphonuclear leukocytes. × 180.

FIG. 4. Higher magnification of a portion of Figure 3. The vacuolated liver cells remain although normal cells have been absorbed. × 300.



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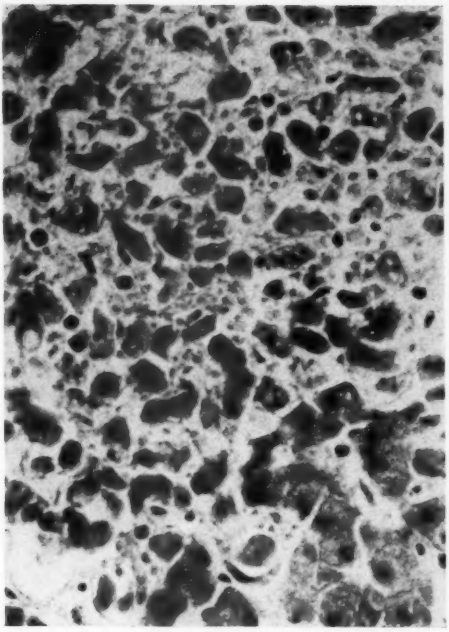

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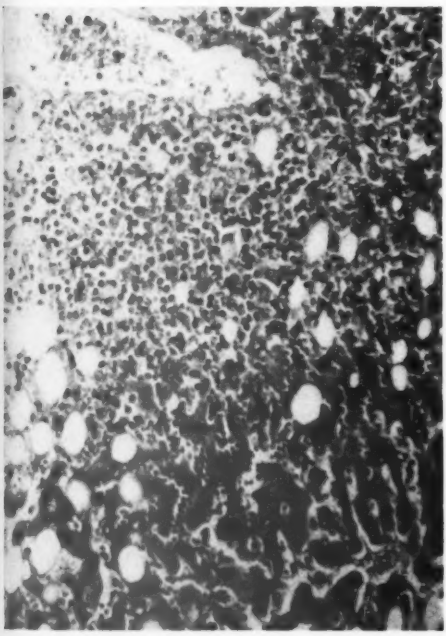

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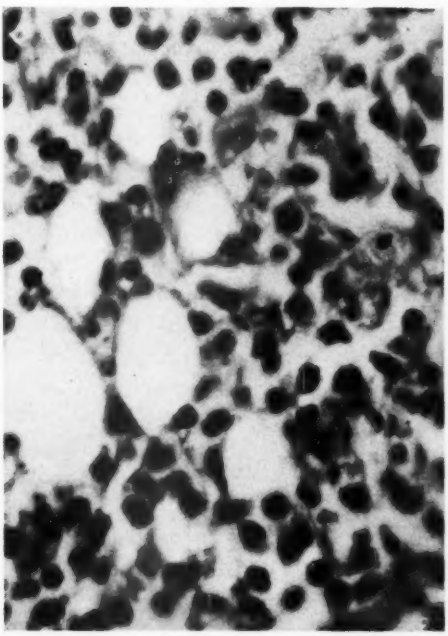

4

Clarke

Centrilobular Hepatic Necrosis

HISTOCHEMICAL STUDIES ON TISSUE ENZYMES
IV. Distribution of Some Enzyme Systems Which Liberate Phosphate at pH 9.2 as Determined with Various Substrates and Inhibitors; Demonstration of Three Groups of Enzymes*

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The development of histochemical methods for the demonstration of alkaline[1-3] and acid[4] phosphatases has made possible the precise localization of these enzymes in various tissues and has yielded information about their possible function.[1-24]

The present study was undertaken to determine relationships among the phosphate-splitting enzymes showing optimal activity at about pH 9.2 and to attempt to correlate some of the earlier chemical data[25-27] with results obtained using these newer technics. It was felt that the potentialities of the histochemical approach were greater than had been realized, and that these methods might be utilized to study the chemical properties of these enzymes in fixed tissues by the same procedures as the biochemist studies them in solution. For instance, the pH optimum of these enzymes may be determined readily by incubating adjacent serial sections of tissues in vessels containing the substrate buffered at varying pH's. The optimum is the pH at which the most intense staining is obtained in a given time. It thus becomes possible simultaneously to determine the pH optima of the phosphatases in different cells, tissues, and organs; studies of this type would be extremely difficult to carry out by the usual methods.

By adopting such procedures and attempting an evaluation of the action of these enzymes when different substrates are employed, and when their action is inhibited by various substances, it has been possible to arrive at a more definite approximation of the number of alkaline phosphatases in various tissues than would have been possible by a strictly morphologic approach. In this study glycerophosphate, glucose-1-phosphate, hexosediphosphate, creatine phosphate, yeast nucleic acid, yeast adenylic acid, thiaminepyrophosphate, barium phytate, muscle adenylic acid, and adenosinetriphosphate have been used.

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The phosphate esters were employed at equimolar phosphorus concentration. Using the different substrates, the enzymes were tested against a series of enzyme inhibitors and activators which included fluoride, cyanide, azide, iodoacetate, glycine, arginine, salts of bile acids, magnesium, ascorbic acid, heat, formalin, trichloroacetic acid, and alcohol.

The results obtained with respect to localization, substrate range, and inhibitor and activator effects suggest that the phosphate-liberating enzyme systems fall into three groups. Group I represents the alkaline phosphatases, previously described by certain investigators.[1-3,5,11] Group I enzymes are demonstrated with all substrates except barium phytate which did not yield inorganic phosphorus at this pH, the most intense staining being observed with hexosediphosphate, muscle adenylic acid, yeast adenylic acid, sodium-beta-glycerophosphate, glucose-1-phosphate, creatine phosphate, and adenosinetriphosphate (Bs), (*cf.* footnote, page 260). Group II enzymes are demonstrated when muscle adenylic acid and adenosinetriphosphate are used as substrates; intense staining is noted in sites which give little or no reaction with the other substrates used for group I. This enzyme is believed to be specific for purine-riboside-5-monophosphoric acids, the characteristic type of linkage in muscle adenylic acid and adenosinetriphosphate, and to correspond to the enzyme reported by Reis[28] to occur in animal tissues in varying amounts. Group III is present in nuclei. Enzymes of this group are readily demonstrated in the nuclei of cells in which neither group I nor group II enzymes are found, but may occur also in association with them. The differentiation of group III from group I is based partly on the observation that muscle adenylic acid, creatine phosphate, sodium-beta-glycerophosphate, and yeast adenylic acid were the most effective substrates for demonstrating nuclear staining. Adenosinetriphosphate (Bs), hexosediphosphate, and glucose-1-phosphate stained nuclei less intensely for a given time of incubation; the latter two substrates are among the best for demonstrating group I enzymes. It is also based in part on the finding that heat,[29,30] dipping in trichloroacetic acid, and incubation in M/4 to M/8 glycine or arginine inhibits the group I enzymes to a much greater extent than the group III nuclear enzymes. The differentiation of group III from group II is based on the observation that creatine phosphate, sodium-beta-glycerophosphate, and yeast adenylic acid stain nuclei as well as muscle adenylic acid (both preparations); whereas sites containing group II enzymes showed little or no staining except with the muscle adenylic acid and samples of adenosinetriphosphate (Bs, A, S), (*cf.* footnote, page 260).

Experimental Procedure

The procedure for the demonstration of alkaline phosphatases as outlined by Gomori[1] is based on the deposition of calcium phosphate at the sites of enzyme activity, when a tissue section is incubated with an organic phosphate ester in the presence of calcium ions buffered at pH 9.2.

Tissues were fixed in cold, absolute acetone for 24 hours using three changes of acetone; the initial fixation was done with the bottle of acetone immersed in a bath of ice water and the following two changes at room temperature. The tissues were next placed in absolute alcohol for 24 hours, followed by 24 hours in toluol, and they were subsequently embedded in paraffin (M.P., 52°). Fixation in cold acetone has the advantage that the same tissue blocks may be used for the demonstration of acid phosphatases[4,10] which are more labile than the alkaline phosphatases. Tissues may be fixed in 95 per cent alcohol if the three groups of alkaline phosphatases are the only enzymes to be investigated.

There have been differences of opinion about the preservation of alkaline phosphatases in various fixatives.[1,31,32] Preservation seems to be mainly a function of concentration of the particular fixatives used, and of the length of time that tissues are kept in a given fixative. Fixation in 4 per cent formaldehyde for 24 hours inactivates the enzymes.[1,3,7] However, when saline,[31,32] pyridine, alcohol, or a number of other substances[31] are added to the formalin and the time in the fixative decreased, quite adequate demonstrations of the enzymes are obtained in frozen sections.

Embedding many tissues in the same paraffin block has the advantages of saving labor and material, of providing comparative data on the relative concentration of the enzymes, and of testing the effect of various substances on the enzymes in different organs.

Following paraffin embedding, sections are taken serially at 10 μ and mounted on slides using glycerin-egg albumin. After cutting and mounting, the slides are dried at 37.5°C. for 24 hours. Some decrease in the intensity of staining was noted when paraffin sections were stored, but sections which had been kept for 6 years showed good staining.

Sections are deparaffinized in two changes of xylol, two changes of absolute alcohol, two changes of 95 per cent alcohol, washed with tap water, and placed into the incubating mixture which is warmed to 37.5°C.

The incubating mixture was freshly prepared for each experiment and consisted of:

10% sodium barbital, 3 cc.
M/10 magnesium chloride, 3 cc.
2% calcium nitrate, 4.5 cc.
Phosphate ester, a weighed amount to give a final concentration of M/300 phosphate.*
Distilled water to a final volume of 30 cc.

The final concentration of barbital is 0.049 M, magnesium 0.01 M, and calcium 0.013 M.

All mixtures were adjusted to pH 9.20 to 9.25 using a glass electrode before being made up to final volume (30 cc.). Sections were incubated for 2, 6, and 24 hours and for 1 week when the effect of a given inhibitor or procedure was to be tested.

To study the inhibiting or activating effects of the various substances mentioned above, a series of Coplin jars was set up, each containing similar amounts of a given substrate, barbital buffer, magnesium, and calcium. Prior to adjusting the pH and total volume, an appropriate quantity of any substance to be tested was added to one of the jars. Adjacent serial sections were incubated for the same time in these solutions and the effect of each substance on the enzymatic activity evaluated by comparison with a section incubated in the absence of this substance.

The relative rate of splitting of the various substrates was evaluated by setting up a series of jars each containing a different substrate and incubating adjacent serial sections for a given time. In all experiments an additional adjacent section was incubated in the absence of substrate to demonstrate preformed calcium.

The sections were removed from the incubating mixture, the pH of the solutions checked with a glass electrode, and the occurrence of

* Samples of sodium-beta-glycerophosphate and yeast nucleic acid were obtained from Eastman Kodak Company, Rochester, N.Y. Another sample of sodium glycerophosphate, a mixture of alpha and beta forms, was obtained from Mallinckrodt & Company, New York, N.Y. Glucose-1-phosphate as the dipotassium salt, creatine phosphate as the calcium salt, and adenosinetriphosphate (Bs) as the dibarium salt were obtained from Bios Laboratories, New York, N.Y. Samples of adenosinetriphosphate as the free acid (S), yeast adenylic acid, yeast nucleic acid, and hexosediphosphate as the barium salt were obtained from Schwarz Laboratories, New York, N.Y. Muscle adenylic acid (Bf) as the free acid was obtained from Bischoff Laboratories, Ivorytown, Conn. Samples of the calcium salt of creatine phosphate,[33] the barium salt of glucose-1-phosphate,[34] and muscle adenylic acid (K) were prepared by one of us (E.A.K.). We wish to thank Dr. Harry G. Albaum for samples of barium phytate and the calcium salt of adenosinetriphosphate (A) and to express our appreciation to Merck & Company, Rahway, N.J., for their gift of thiaminepyrophosphate.

precipitates noted. The deposited calcium phosphate indicative of the extent of enzyme action was visualized by the von Kossa procedure as previously described.[1,5]

There are several alternative methods for the final visualization of the calcium phosphate[1]: one method involving the use of cobalt with the final product being cobalt sulfide; the other the von Kossa method, the final precipitate being free silver. The problem of the affinity of various cell components for cobalt will be discussed later.[1] Most of the workers in the field have used the cobalt salt as the method of visualization.

The effects of substances on phosphatase activity were evaluated by comparing grossly and microscopically the intensity of staining of serial sections, incubated in the presence of the given substance, with a comparable control section incubated in a solution not containing the substance. It is of interest that in those jars containing substrates without any inhibitor and in which enzymatic action occurred, the solutions after incubation were found to have dropped as much as 0.7 to 1.0 pH unit; in those jars containing concentrations of substances which inhibited enzymatic action, such as glycine or cyanide, little or no change in pH was observed after the sections were removed; with substances which enhanced phosphatase activity, *i.e.*, ascorbic acid, the final pH was even lower than in the control solutions. It is evident that this change in pH would tend to decrease the rate of the enzymatic reaction.

The von Kossa procedure transforms the precipitated calcium phosphate into silver phosphate, which is then reduced to metallic silver. The appearance varies from granular to crystalline and the color from brown to black. With most substrates, the precipitate is dark brown and granular. Some of the substrates are fairly characteristic with respect to the appearance and color of the final precipitate. Sections incubated in hexosediphosphate tend to show a smudged yellow-brown precipitate. With thiaminepyrophosphate, the precipitate is usually arranged in a unique star-shaped figure. With nucleic acid, thiaminepyrophosphate, and some samples of muscle adenylic acid and adenosinetriphosphate, the precipitate is crystalline and coats or outlines the involved structures much less precisely than does the granular form. With different samples of the same substrate, some variations in the resulting silver precipitate were noted, although the sites of localization were the same. Thus, with muscle adenylic acid (Bf) the precipitate is often crystalline, while with the other sample

the precipitate tends to be granular. Similarly, adenosinetriphosphate (Bs) often results in a granular precipitate, while adenosinetriphosphate (S) consistently gives a crystalline precipitate. It must be emphasized that these observations reflect a general tendency and that some variation may frequently occur. Occasionally, sections of two organs on the same slide may vary in the character of the precipitate. The factors involved remain obscure. One is almost certainly the rate of deposition of the original calcium phosphate precipitate, which would vary with the rates of hydrolysis of the individual substrates by the enzymes. This might result in differences in crystal size and configuration which might influence the character of the subsequently formed silver phosphate and metallic silver deposits. With some substrates, notably adenosinetriphosphate and thiaminepyrophosphate, it is possible that the enzymatic action liberates pyrophosphate, rather than phosphate, which then precipitates as calcium pyrophosphate. With these two substrates, it was noted also that more intense staining was obtained with shorter incubation times (2 hours) and that increased times (6 to 24 hours) frequently resulted in a decrease in the amount of precipitate obtained. Morse and Greep[35] reported a similar finding with certain tissues when glycerophosphate was used as substrate, but this is contrary to our experience using glycerophosphate or substrates other than thiaminepyrophosphate and adenosinetriphosphate. The nature of this phenomenon is not clear.

It is evident that the precipitated silver marks the location of the sites of enzyme activity. The possibility has often been considered that some diffusion of the precipitate of calcium phosphate, and of silver phosphate in the subsequent processes, might occur. Danielli[31] ingeniously demonstrated that diffusion does not occur, at least from one section to another applied directly over it. In this study it has been noted that within some organs there is a tendency for some cells immediately adjacent to an intensely affected structure to show more precipitate than do morphologically similar cells farther removed. Danielli[31] believed that he had demonstrated in skin and kidney, that those structures which contain alkaline phosphatases also show non-specific affinity for calcium phosphate. His observations are probably attributable to his failure to inactivate nuclear enzyme in heated sections completely, so that the calcium phosphate which he observed in nuclei was probably the specific result of enzyme which survived the heating process. No evidence of non-specific deposition of calcium phosphate was noted when tissue sections were incubated for as long as 1 week in a saturated solution of calcium phosphate.

With periods of incubation of substrate mixtures up to 1 week, a uniform, diffuse, finely granular precipitate is sometimes noted throughout the sections and in some instances on the glass slide between sections. This is the result of enzymatic action since it does not occur when enzymatic action is inhibited. However, it does not localize the sites of enzyme action and has been disregarded. This non-specific deposition is probably the result of small quantities of enzyme from the sections diffusing out into the solution and of precipitation of calcium phosphate uniformly over the section as a consequence of the action of enzyme in solution. In an experiment in which a solution of rat intestinal phosphatase prepared by autolysis[36] was added to the incubating medium containing the sections, a striking increase in such uniform non-specific deposition was noted. Indeed, under these circumstances non-specific deposition could be produced after 6 hours of incubation, appearing first and most intensely when the concentration of enzyme in solution was highest.

Dialysates of preparations of rat intestinal or guinea-pig kidney and adrenal phosphatases,[36] prepared by autolysis using twenty times as much distilled water as tissue, were added to various incubating mixtures in final concentrations of as low as 1:100 to 1:1000 of the original autolysates. A marked enhancement of all three groups of enzymes and especially of nuclear staining was noted; effects on cytoplasmic staining were less conspicuous. The activating effect[37] could also be demonstrated with undialyzed solutions of phosphatases which had been boiled for 30 minutes. The activating substance is probably not magnesium ion, since its effect is maintained in the presence of optimal activating concentrations of magnesium, and it is probably not glycine for the same reason. When an inhibiting concentration of glycine is used (M/4), the activating effect of the dialysates is still evident, again suggesting that it is not glycine.

Occasionally, with longer periods of incubation, 24 hours to 1 week, the entire section, with the possible exception of the red cells, lost its affinity for the eosin used as a counterstain. This phenomenon has not been investigated. In such instances, precipitate representing enzymatic activity is noted in some of the same areas where it is present on sections which have not lost their eosinophilic properties, but such sections may show less definitive staining.

Material

The organs of a group of freshly killed healthy animals, and normal and abnormal tissues from human autopsies and experimental animals

were examined. The species included guinea-pig, rat, rabbit, mouse, and man.

Description

The descriptions which follow are based on the examination of approximately 4300 slides, most of which contained more than one tissue. In most instances the descriptions represent the summation of a number of observations, so that the more consistent effects of enzymatic activity and of inhibitors and activators are given, without reference to the variations inherent in the method. In other instances, a given period of incubation, one or another substrate, or the action of a given concentration of an inhibitor was not available. No specific reference is made to these occasions, since the effects could be inferred from ancillary data; for example, in a given tissue, the effect of M/4 glycine as an inhibitor may be taken for granted if M/4 arginine was noted to give the characteristic amino acid effect. The number of permutations in terms of the factors investigated is large and it is not to be assumed that every possible combination of experimental conditions has been examined in the present study.

Attention is called to the general excellence and completeness of the initial descriptions of the localization of enzyme and of species variations in enzyme distribution as recorded by Gomori[1,3] and Bourne[11] using glycerophosphate as substrate with periods of incubation of from 2 to 3 hours. With similar periods of incubation the present investigation confirms these descriptions. Longer periods of incubation indicate that enzyme activity is present at sites previously reported as negative.[1,3,5,7,11] The results obtained in this laboratory with different substrates and inhibitors have led to an interpretation differing from those previously advanced.[22,38-41]

The substrates sodium-beta-glycerophosphate, glucose-1-phosphate, creatine phosphate, yeast adenylic acid, yeast nucleic acid, hexosediphosphate, and thiaminepyrophosphate will be called group A, while muscle adenylic acid and adenosinetriphosphate will be referred to as group B.

In recording the histologic appearances following the use of these two groups of substrates, a complete description is first given of the findings with group A; references to the descriptions of others are given in parentheses. The results with group B substrates are then appended but only when additional structures demonstrating enzyme activity are noted, since all the sites of activity reported for group A substrates are duplicated with group B.

Heart. With short incubation periods in group A substrates, staining of heart was limited to capillary endothelium in the rabbit[42] and guinea-

pig.[11,42] In the rat, there was additional staining of the adventitia of larger blood vessels[3] and of some muscle nuclei. In the mouse, capillary endothelium[5,42] and muscle nuclei stained. There also was faint granularity of muscle fibers. With longer incubation, all capillary endothelial cells of guinea-pig heart stained deeply (Fig. 3), and there was a light granular precipitate in muscle fibers. In the rabbit (Fig. 1), and man, there was in addition slight staining of muscle nuclei, and in the rabbit, fibroblast nuclei also were evident. With group B substrates no differences were noted in mouse and rat heart, while human, rabbit (Fig. 2), and guinea-pig heart (Fig. 4) showed intense staining of muscle fibers of the media of coronary arteries.

Lung. With group A substrates and short incubation periods there was staining of many nuclei of the septa, bronchial epithelium, and of a few nuclei of vascular endothelium of the lung of the guinea-pig.[3,11] Cytoplasm stained in the basal portions of bronchial cells. In the mouse[3,5] and rat, there was additional staining of the cytoplasm of septal cells. Cytoplasm of mouse capillary endothelium and the outer edge of the adventitia of pulmonary vessels in the rat[3] also stained. Data after short periods of incubation in rabbit[3] and man[5] were not available. With longer incubation there was additional staining of nuclei of cartilage cells and of nuclei of bronchial smooth muscle in the guinea-pig. In the rat there was added light staining of bronchial epithelium. In the rabbit, some septal nuclei and the nuclei and cytoplasm of epithelium were demonstrated. Some nuclei of alveolar septa, of the vascular endothelium, and the smooth muscle of larger blood vessels, stained in man. With group B substrates, no differences in sites of staining with tissues from rat, mouse, and man were noted. In the guinea-pig and rabbit there was intense staining of nuclei, and of smooth muscle fibers of the media of pulmonary vessels.

Trachea. In the trachea of the rabbit,[3] with group A substrates for short periods of incubation, light staining of the brush border of the mucous membrane, moderate staining of nuclei of epithelial cells which was more marked basally, and light staining of occasional nuclei of stroma and blood vessel walls were observed. In the guinea-pig[3,11] the cytoplasm of epithelial cells stained lightly and the nuclei and brush border of submucosal glands also stained lightly. With longer incubation, more nuclei, including nuclei of cartilage cells, were demonstrated in the rat. In the guinea-pig, there was moderate to deep staining of both nuclei and cytoplasm in the epithelial layer and occasional staining of some nuclei and capillaries in the submucosa. With group B substrates, in the rat and guinea-pig the nuclei and cytoplasm of the muscular layer and of the media of blood vessels in the submucosa

were stained as well. In the latter animal, the nuclei and cytoplasm of cartilage cells, and the cartilaginous matrix, which are unstained with group A substrates, showed staining. Material was not available for the other species.

Spleen. Incubation of spleen for short periods in group A substrates in the guinea-pig[3,11] led to staining of a few scattered nuclei in malpighian follicles and in pulp, and of cytoplasm of short segments of sinusoidal endothelium. In the mouse[5] there was more consistent staining of endothelium and staining of lymphocytic nuclei at the periphery of follicles. With longer incubation, guinea-pig spleen showed additional staining of lymphocytic nuclei at the periphery of follicles, of fibrocytes of the capsule and trabeculae, and of the media of blood vessels. This was observed for mouse, rabbit, and man, as well. In the rat, the only difference was that follicles were stained very little, with some staining of capsular fibers and of muscle fibers of the media of larger blood vessels. With group B substrates, in the guinea-pig, mouse and man there was intense staining of cytoplasm of the media of larger blood vessels, and in the mouse and man, of fibers of trabeculae.

Liver. Sections of guinea-pig liver incubated for short periods in group A substrates revealed staining of nuclei of Kupffer cells.[3,11] In the rat[3,38] the cytoplasm of endothelial cells also was stained. In the mouse,[5,38] cytoplasm of endothelial cells in scattered sinusoids and in some capillaries, and nuclei of occasional hepatic cells near central veins stained as well. In the rabbit[3] the appearance was like that in the mouse except that nuclei of fibrocytes in portal areas and of cells lining bile canaliculi were stained. The findings in man[3,5,23] (Fig. 65) were essentially similar. With longer incubation, all structures mentioned were stained in all species. Incubation of guinea-pig liver with group B substrates resulted in staining of the cytoplasm of some vascular endothelium and of smooth muscle in blood vessels and larger bile ducts. In the rat the cytoplasm of bile canaliculi also was stained.

Gallbladder. With group A substrates for short incubation periods in the gallbladder of the guinea-pig,[11] nuclei of fibrocytes in the lamina propria and nuclei and cytoplasm of capillaries were stained. With longer incubation, moderate staining of epithelial nuclei and slight staining of epithelial cytoplasm were noted. With these longer periods of incubation, human gallbladder showed staining of bile, nuclei of epithelium, and some nuclei and perhaps collagen fibers in the remainder of the wall. With group B substrates no additional structures were stained in man, while in the guinea-pig, nuclei and cytoplasm of smooth muscle fibers were stained. Data were not available for the other species.

Pancreas. Guinea-pig pancreas, incubated for short periods in group A substrates, appeared like that described by other investigators.[3,11] Only the capillaries and small ducts were visualized. With longer incubation, these structures stained more intensely and consistently. In addition, there was considerable impregnation of nuclei and to a lesser degree of cytoplasm in the acinar and islet cells. The nuclei of fibrocytes and smooth muscle fibers of blood vessels stained well. The pancreas of the rabbit and man were similar to that of the guinea-pig.[3] Rat pancreas differed in that the peripheral islet cells stained somewhat more deeply than did the central islet and the acinar cells.[3,38] Rat and mouse also differed from the others in that the media of arteries stained. Use of group B substrates led to staining of the media of the muscular arteries and veins in the guinea-pig, rabbit, and man. The increased staining of peripheral islet cells in the rat was less apparent.

Salivary Glands. With group A substrates and short periods of incubation of salivary glands of the mouse,[38,43] capillary endothelium and the adventitia of larger blood vessels stained. Nuclei of cells of both types of glands stained, those of mucous glands more deeply than those of serous glands. For the rat,[3,43] findings were similar, with the addition of staining of the basement membrane of the glands and some nuclei of the ducts. For the guinea-pig,[3,11] staining was limited to capillary endothelium and a few pericapillary nuclei. With longer incubation, some duct nuclei were now demonstrated in the mouse,[38,43] but no additional structures stained in the rat.[38,43] In the guinea-pig, many nuclei were stained, those of ducts more darkly than those of alveoli. With group B substrates, no differences in localization were noted for the mouse and rat. For the guinea-pig, the nuclei and cytoplasm of blood vessels stained.

Esophagus. With group A substrates for short incubation periods of the esophagus of the rabbit, there was moderate staining of epithelial nuclei, more marked in the basal layers, as well as of scattered nuclei in the submucosa. With longer incubation, additional nuclei were stained in all layers, including the muscular layer, and nuclei of all blood vessels were demonstrated. For the guinea-pig under similar circumstances, the nuclei and cytoplasm of the basal layers of the epithelial lining stained, as did nuclei in the portion of the submucosa contiguous with the epithelium. Capillary endothelium and pericapillary nuclei of muscle fibers were demonstrated also in the muscular layer. With group B substrates there were no additional structures stained in material from the rabbit, while in the guinea-pig, nuclei and cytoplasm of the muscular layer were consistently stained.

Stomach. For the guinea-pig,[3,11] using short periods of incubation with

group A substrates, capillary endothelium in the outer half of the gastric mucous membrane stained. In the rabbit,[3] endothelium of scattered capillaries and nuclei of occasional cells in the lamina propria and of endothelium also were stained. With longer incubation of the stomach of the guinea-pig, capillaries in the muscular layer and nuclei in mucous membrane stained moderately to deeply, while cytoplasm of mucous membrane stained lightly. In the rabbit, staining was darker and some additional nuclei were observed. For man, for these longer periods, capillary endothelium stained in all layers, and nuclei of fibrocytes in the serosa were demonstrated. There was slight granularity in cytoplasm of epithelial cells. With group B substrates, cytoplasm and nuclei of the muscular layer stained deeply in the stomach of guinea-pig and man. For the rabbit, only an external and internal band of the muscular layer stained in this fashion. The media of blood vessels in the rabbit also stained deeply. Data on this point were not available for the other species.

Small Intestine. With group A substrates and short incubation periods of the intestine of the guinea-pig,[3,11] the endothelial lining of capillaries in all layers stained occasionally and moderately. The brush border of the mucous membrane stained deeply in some places. In the rabbit[3] (Fig. 61), capillary endothelium in the muscular layer, nuclei and cytoplasm in the lamina propria, and occasional nuclei in all layers except the muscularis were stained. In the rat,[3] capillary endothelium throughout the wall and nuclei and cytoplasm of smooth muscle cells in patches in the internal muscular layer were stained. Brush border, nuclei, and cytoplasm of the lamina propria and cytoplasm of the internal muscular layer were stained in the intestine of the mouse.[5,22,44] With longer incubation, the structures demonstrated in the guinea-pig (Figs. 5 and 57) were stained more deeply and consistently and, in addition, there was irregular staining of epithelial nuclei. In the rabbit, there was additional staining of nuclei of the muscular layer. In the rat, some epithelial nuclei and many nuclei of the muscular layer and adventitia were demonstrated. In the mouse,[22] additional nuclei were seen in epithelium and in other structures. Staining of intestinal contents was noted occasionally.[3,5] With group B substrates for the guinea-pig (Figs. 6 and 59), the muscular layers including the muscularis mucosae showed consistent staining of nuclei and cytoplasm. In the rat, staining of nuclei and cytoplasm was uniform and intense in all muscular layers, including the muscularis mucosae. In the rabbit, patchy staining of cytoplasm of the internal and external muscular layers and staining of the entire wall of larger

blood vessels were observed. In the mouse, staining of nuclei and cytoplasm of the external muscular layer was noted.

Large Intestine. Group A substrates and short incubation periods for the large intestine of the rat[3] showed capillary endothelium to stain deeply and occasional nuclei in the mucous membrane to stain lightly. In the internal muscular layer, both nuclei and cytoplasm stained. In the mouse,[5] the brush border of the epithelium stained markedly, in addition. In the rabbit,[3] additional structures, lightly stained, were nuclei and cytoplasm of the lamina propria. With longer incubation periods for the rat and rabbit, staining was similar but more intense, while for the mouse, staining of additional nuclei in the mucous membrane and muscular layer was noted. For the guinea-pig,[11] with such longer incubation, capillary endothelium stained throughout, while epithelial nuclei stained moderately and epithelial cytoplasm lightly. For man,[5] the structures demonstrated were similar to those of the mouse, with additional staining of some serosal nuclei and fibers. Some staining of intestinal contents occasionally was noted.[3,5] With group B substrates, staining also occurred in the internal and external bands of the muscular layer of the guinea-pig, mouse, and man. For the guinea-pig, the media of muscular blood vessels stained in addition, and in all of these structures both nuclei and cytoplasm of smooth muscle were demonstrated. The rat and rabbit showed no staining beyond that noted with group A substrates.

Kidney. The appearance of sections of guinea-pig kidney incubated for short periods of time in group A substrates was essentially like that previously described.[1,3,6,11] The greatest staining occurred in the brush borders of the proximal convoluted tubules, with moderate staining in the nuclei of these structures. With longer periods of incubation (Figs. 7 to 13 and 55), the structures first stained were more deeply impregnated. In addition, the basement membranes of the proximal convoluted tubules and the nuclei of other segments of the tubule and of the glomeruli were stained. The nuclei of the distal portion of the tubular system required the longest periods of incubation. Nuclei in the capsule and stroma stained lightly, as did those in the collecting tubules. Rat,[3,6,12,15] mouse[5] (Fig. 44), rabbit,[1,3,12] and human[3,5] kidneys were essentially similar to guinea-pig kidney except for moderate staining of the cytoplasm of glomeruli and of the adventitia of the muscular arteries and veins in the mouse and rat even for short periods of incubation.[3] With group B substrates (guinea-pig, Figs. 14 and 15), there was staining of the media of the muscular arteries and veins of the guinea-pig, rabbit, and man.

Ureter. With group A substrates and short incubation, nuclei and cytoplasm of ureteral epithelium and capillaries stained in the mouse (Fig. 17), rabbit,[3] and rat.[3] For the rabbit some nuclei stained in the submucosa. For the mouse faint nuclear staining occurred in the muscular layer, while for the rat both nuclei and cytoplasm of the muscularis stained deeply. With longer incubation periods no additional structures were stained in material from the mouse and rat. No data were available for the rabbit. With group B substrates no additional structures were stained for the rabbit or rat while for the mouse (Fig. 18), nuclei and cytoplasm of the muscular layer were stained.

Urinary Bladder. With group A substrates and short incubation of the bladder of man[3] and guinea-pig,[3,11] there was moderate staining of nuclei of mucosal epithelial cells and of connective tissue cells of the submucosa. There was light staining of cytoplasm of epithelial cells. For the other species, the same structures were demonstrated, with more intense staining of the cytoplasm of the epithelial cells of the mouse, rat,[3,11] and rabbit.[3] For all of these species, capillary endothelium was stained, but incompletely so in the guinea-pig. In the rat the muscular layer showed patchy staining of muscle fibers and nuclei, and in the rabbit the band of muscular layer just beneath the mucous membrane showed staining of both nuclei and cytoplasm. In the guinea-pig and mouse there was no staining of the muscular layer. With longer incubation periods the same structures stained in the same species (guinea-pig, Figs. 21 to 25), but with more nuclear staining in the muscular layer. With group B substrates, all muscular cytoplasm of the guinea-pig (Figs. 26 and 27), man, and mouse was deeply stained. In the rabbit, an external band of muscle fibers stained in addition to the internal band stained with group A substrates. In the guinea-pig and man, there was staining of muscle fibers of the media of blood vessels. In the rat, the findings were similar to those obtained with group A substrates.

Testis. With short incubation periods and group A substrates, the testis of the guinea-pig[3,11] stained in endothelial cells of capillaries, in the fibers of the capsule, and in cytoplasm of some of the cells of the tubules. The nuclei of tubular and interstitial cells stained lightly. In addition, in the mouse[45] and rat,[3] the basement membranes of the tubules and the adventitia of blood vessels stained. Only nuclei of the basal portion of the tubular epithelium stained and the cytoplasm of tubular cells was unstained. For the rabbit,[3] marked staining of nuclei and cytoplasm of tubular cells and of nuclei of interstitial cells was observed. For man,[5] there was light staining of nuclei of interstitial

cells, moderate staining of nuclei of cells of tubules, and deep staining of capillary endothelium. With longer incubation periods the structures stained were similar in all species. With group B substrates, there were no differences in sites of staining in the mouse, rat, or rabbit. In the guinea-pig, muscle fibers of the media of blood vessels were stained, and in man, fibers of the tunica albuginea were demonstrated. No larger blood vessels were seen in the specimens from man.

Epididymis. With group A substrates and short periods of incubation the basement membrane and capillary endothelium of the epididymis of the guinea-pig[3] stained regularly. In the mouse,[42] not only did the basement membrane stain, but also nuclei and cytoplasm of the stroma. In the rabbit,[3] nuclei and cytoplasm of epithelial cells stained variably. In one well defined zone, cytoplasm stained darkly, more so toward the lumen, and nuclei stained lightly to moderately. In the other well defined area, cytoplasm stained slightly and nuclei lightly. In the rabbit,[3] the findings were similar to those in the mouse except for absence of staining of cytoplasm of stromal cells. With longer incubation periods there was staining of some nuclei of the basal layers and of fibrocytes of the stroma. No additional structures were stained in the mouse[42] (Fig. 20). In the rabbit, the difference in depth of staining of the cytoplasm of epithelial cells in the two areas referred to above persisted, but was less marked. In the rat, for these longer periods, nuclei and cytoplasm of vessels, nuclei of epithelial and stromal cells, and the basement membrane of epithelium stained. With group B substrates no differences were observed.

Vas Deferens. For the rabbit,[42] with group A substrates for short periods of incubation, an occasional nucleus of an epithelial cell or of a cell in the lamina propria of the vas deferens stained. For longer incubation periods, additional nuclei stained in all layers. In the rat,[42] nuclei of epithelial cells and nuclei and cytoplasm of the muscular layers stained. In the guinea-pig,[11,42] the picture was the same except that staining of the muscular layers was patchy. With group B substrates, no additional structures were stained in the rabbit or rat. In the guinea-pig, all of the muscular layer stained.

Seminal Vesicles. With group A substrates for short incubation periods for the guinea-pig,[11] there was marked nuclear and cytoplasmic staining of epithelial cells and of capillary endothelium of the seminal vesicles. In the mouse, the basement membrane stained deeply, epithelial nuclei lightly, and nuclei and cytoplasm of the muscular layer deeply. The endothelium and adventitia of blood vessels stained moderately. In the rabbit, the findings were like those in the guinea-pig

with the additional staining of a few nuclei in the lamina propria. With longer incubation, no additional structures were stained in the seminal vesicles of the guinea-pig (Fig. 28), or mouse (Fig. 19). In the rabbit, nuclei of the muscular layer also stained. Staining of luminal contents was erratic, varying from unstained to moderately stained in the same section. No data were available for the other species.[5] With group B substrates, the nuclei and cytoplasm of the muscular layer were stained in the guinea-pig material (Fig. 29), while for the mouse and rabbit the picture was essentially unchanged.

Ovary. With group A substrates and short incubation periods for the ovary of the guinea-pig,[3,46] staining was observed in nuclei of serosal cells, of fibrocytes of the capsule, of granulosa cells, of cells of the theca interna, and to a lesser extent of the theca externa of endothelial lining of blood vessels. There was fine granular staining in fibers of the capsule and in cytoplasm of the granulosa and theca cells. In the mouse (Fig. 41), a few nuclei of stroma and of granulosa cells, cytoplasm of endothelial cells of capillaries and theca cells, and patchy areas in the stroma stained moderately to deeply. When longer incubation periods were employed, these same structures stained more darkly and consistently. In the rat,[3] incubation for longer periods resulted in staining of capillary endothelium, adventitia of larger blood vessels, nuclei of granulosa, theca and stromal cells, and cytoplasm of theca cells and, to a lesser degree, of granulosa cells and some fibers in the stroma. There were no observations on this material for rabbit[3] and man.[3,46] With group B substrates, no differences were observed in the guinea-pig and mouse. In the rat, all stroma showed consistent cytoplasmic staining. Data were not available for the other species.

Ovarian Tube. With group A substrates and short incubation of the ovarian tube of the mouse, the brush border of the epithelial lining, cytoplasm and nuclei of epithelial cells, nuclei and cytoplasm of capillary endothelium in the lamina propria, and nuclei and cytoplasm in the muscular layer were stained. Staining of muscle was deeper externally. In man, staining was limited to nuclei and cytoplasm of capillaries and epithelium. With longer incubation no additional structures stained in the mouse; while in man, nuclei of fibrocytes and smooth muscle cells were demonstrated also. In the guinea-pig, with these longer incubation periods, the brush border of the epithelial lining, nuclei, and cytoplasm of capillary endothelium in the muscular layer stained, and there was slight staining of nuclei of the epithelium. In the rat, the nuclei of the epithelial cells stained somewhat more deeply and there also was staining of nuclei and cytoplasm of the muscular

layer, more marked just beneath the epithelium. Data for the rabbit were not available. With group B substrates, nuclei and cytoplasm of the muscular layer in the rat stained deeply and uniformly, while in the guinea-pig and man, nuclei and cytoplasm of the muscular layer, which were unstained with group A substrates, stained deeply. No additional structures stained in the mouse.

Uterus. With group A substrates for short periods of time, the uterus of mouse[47,48] and man[47,49] showed staining of the nuclei and cytoplasm of the cervical epithelium, while in the fundus, the free border also stained. The endometrium showed staining of nuclei, while in the cervix the stroma showed some patches of cytoplasmic staining in the mouse, but little or none in man. Nuclei of muscle cells of the myometrium and of capillary endothelium were demonstrated also in these species and in addition, cytoplasmic staining was noted in the myometrium of the mouse. In the rat,[3] the epithelial lining and endometrial stroma stained very much like those in the mouse, while the myometrium remained unstained except for uniform capillary staining. With longer incubation, tissues of the mouse and man showed no additional staining, while those of the rat (Figs. 30 to 36) exhibited faint staining of nuclei of the myometrium. The guinea-pig uterus[3] stained similarly to that of other species, except for staining of the cytoplasm in the endometrial stroma and occasional patches of subserosal cytoplasm in the myometrium. With group B substrates the mouse showed no staining of additional structures, while in the rat (Figs. 37 to 40), man, and guinea-pig, nuclei and cytoplasm of the myometrium were deeply stained. (The description of the findings in the uterus was not complete since insufficient specimens were examined; these did not cover the entire estrus cycle,[24,47,48] nor were differences dependent upon age considered in the present material.)

Breast. With group A substrates for short incubation periods in man[5,39] and guinea-pig,[39] nuclei and cytoplasm of mammary alveolar and ductal epithelial cells were deeply stained. With longer incubation, nuclei of stromal cells and of the walls of blood vessels also were stained. No data were available for the other species. With group B substrates, the only differences observed were in the walls of blood vessels, where the cytoplasm now stained in both species.

Adrenal Gland. With short periods of incubation in group A substrates, the guinea-pig adrenal gland showed intense staining of the cytoplasm of cells of the glomerular layer.[3,11] The cytoplasm of capillary endothelium and occasional nuclei in the glomerular layer stained to a lesser degree. With longer incubation (Figs. 47 and 49), these struc-

tures stained more intensely and nuclei stained more deeply and in greater numbers. This increased nuclear staining usually progressed from without inward. In the rabbit,[8] the glomerular layer, and in man, the subcapsular portion of the glomerular layer, showed little or no staining of nuclei or cytoplasm. Human adrenal gland[3] exhibited marked staining of the cytoplasm of cells in the remainder of the cortex with lesser staining of nuclei. In the rabbit,[3] cytoplasmic staining was minimal and nuclear staining in the remainder of the cortex was moderate. Rabbit medulla showed very slight nuclear and cytoplasmic granularity. In the mouse,[5] the nuclei of cells of the cortex stained moderately and their cytoplasm stained lightly. Nuclei of cells of the medulla required longer periods of incubation for their demonstration. The entire wall of blood vessels stained. In the rat,[3] nuclei in the cells of the cortex stained more definitely than did the cytoplasm, except for the outer portion of the glomerular layer in which the cytoplasm was better impregnated. The capillary network by comparison stood out as sharply stained.[3,11] With group B substrates there was, in addition, intense staining of the media of the muscular arteries and veins in the guinea-pig.

Thyroid Gland. With group A substrates for short periods of incubation in the rabbit,[8] nuclei of epithelial cells stained darkly and cytoplasm stained lightly or not at all. Colloid did not stain. In the guinea-pig[11] and man,[3] only capillary endothelium stained. With longer incubation periods, nuclei stained more deeply in the rabbit; in the guinea-pig and man, nuclei stained moderately and cytoplasm lightly or not at all. In man, staining of colloid was erratic and varied from unstained to moderately stained on the same slide. With group B substrates the thyroid glands of rabbit and man showed no additional staining. Data were not available for the guinea-pig.

Pituitary Body. With group A substrates and short incubation, nuclei of anterior lobe cells and of pituicytes of the posterior lobe stained lightly in man.[5] Capillary endothelium stained moderately and occasional patches of cytoplasmic staining were noted in the capsule. With longer incubation the structures stained more darkly and consistently, and fibrocyte nuclei in the anterior lobe stained moderately. No data were available for the other species.[11,50] With group B substrates, more consistent staining was noted in the capsule, and, in addition, the interstitial substance of the posterior lobe was stained.

Pineal Body. With group A substrates and short incubation, nuclei and cytoplasm of capillaries of the pineal body, and some nuclei of parenchymal cells were stained in the rat. With longer incubation in the

guinea-pig the picture was the same. Data were not available for other species.[50] With group B substrates, no additional structures were stained in the rat or guinea-pig.

Thymus. With group A substrates and short incubation in the guinea-pig, only scattered nuclei of thymic cells were seen. With longer incubation, observations were available only in the rabbit,[3] which showed patchy staining of nuclei of thymic cells. With group B substrates, no differences were observed in the guinea-pig, but in the rabbit, staining of capillary endothelium was seen. The observations were few and may not represent the true state of affairs.

Lymph Node. With group A substrates for short periods of time in the lymph nodes of guinea-pig, mouse, man,[5] and rat, capillary endothelium and some nuclei, probably those of reticulo-endothelial cells and of some lymphocytes, stained. No data were available for the rabbit. With longer incubation periods, the structures mentioned stained more deeply and consistently. With group B substrates, the findings were similar except for additional staining of nuclei and fibers of the capsule in the guinea-pig and of additional nuclei in the mouse.

Skeletal Muscle. With group A substrates for short periods, the capillary endothelium of muscle stained in guinea-pig,[11,42] mouse,[5,42] rat,[42] rabbit,[42] and man.[5,42] With longer incubation, capillaries stained more consistently and there was slight granularity of muscle fibers. Some nuclei of muscle stained in all species but the mouse. With group B substrates, no differences in staining were noted except in the mouse in which muscle nuclei stained.

Skin. With group A substrates for short incubation periods in man,[51] nuclei and cytoplasm of basal cells of the epidermis, of sweat glands, and of capillaries were stained. With longer incubation, nuclei of fibrocytes and of the arrectores pilorum muscles also stained. Data for other species were not available.[11,21] With group B substrates, there was, in addition, staining of cytoplasm of the arrectores pilorum.

Nervous System

Neural Parenchyma. With group A substrates and short incubation periods, there was deep staining of nuclei and cytoplasm of capillary endothelium of the neural parenchyma of the guinea-pig,[3,11] mouse,[7] rat,[3] and man[3,7]; while in the rabbit,[3] this appeared to be less consistent. Nuclei of nerve and glial cells stained lightly, interstitial substance of gray and white matter moderately, but more intensely in the gray matter, and the external glial membrane moderately in all parts of the nervous system examined. Nerve fibers stained lightly on

occasion. This was never as consistent, intense, or complete as was staining of axons for acid phosphatase.[10] In man, all of these additional structures remained unstained while corpora amylacea showed some staining.[7] With longer incubation periods, no additional structures were stained except in human tissues (Fig. 51), in which some nerve and glial cells and interstitial substance of the gray and white matter stained lightly. With group B substrates, interstitial substance stained much more deeply in all of the species examined. In the rat and rabbit, staining was equal in gray and white matter; in mouse and guinea-pig, gray matter was darker than white matter. In man, staining of interstitial substance became deep, with gray matter darker than white matter; nuclei of nerve and glial cells stained moderately, cytoplasm of nerve cells stained lightly, and the media of larger blood vessels also were stained.

Ependymal Lining. For short incubation periods with group A substrates, nuclei of ependymal cells of guinea-pig, mouse,[7] rat, and rabbit stained lightly. With longer incubation, no additional structures were stained. With group B substrates, the picture was essentially the same.

Choroid Plexus. With group A substrates for short incubation periods, nuclei and cytoplasm of capillary endothelium of choroid papillae stained deeply for the guinea-pig, rat, and mouse,[7] while choroid lining cells were like those in the ependyma except for deeper staining. With longer incubation the findings were essentially the same. With these longer periods of incubation the choroid plexus in the rabbit resembled that of the other species. No additional structures were stained when group B substrates were used.

Leptomeninges. With group A substrates for short incubation periods, nuclei and cytoplasm of arachnoid cells were stained in the species examined. Nuclei and cytoplasm of these cells were deeply impregnated in the rat, mouse,[7] and man,[7] and were lighter in the guinea-pig and rabbit. Nuclei of fibrocytes stained moderately in the rat and the adventitia of larger leptomeningeal vessels stained in the mouse. With longer incubation, the only difference was the staining of occasional pial nuclei and occasional smooth muscle nuclei in the walls of large blood vessels in man. With group B substrates, no additional structures were stained in the mouse and rat. In the guinea-pig, rabbit, and man, the media of larger blood vessels showed staining of nuclei and cytoplasm; in the rabbit, nuclei and cytoplasm of pial cells stained more deeply and consistently, and in man the adventitia of blood vessels and connective tissue fibers in the pia and arachnoid also were stained.

Nerve Roots. With group A substrates for short periods of time,

capillaries of nerve roots stained moderately and axons lightly in the rabbit. With longer incubation, axons stained moderately. With group B substrates, axons stained deeply in this species.

Nerves in Viscera. With group A substrates for short periods of incubation, there was light staining of axons in the mouse, guinea-pig, and rabbit. With longer incubation, axons and epineurium stained lightly in the mouse and deeply in the rabbit, while in the guinea-pig there was, in addition, a uniform granular deposit throughout the nerve trunk which tended to obscure axonal staining. With group B substrates the diffuse granular deposit in the guinea-pig appeared earlier and was deeper, while no differences were observed in the mouse and rabbit.

Visceral Ganglia. With short incubation periods, using group A substrates, the nuclei of ganglion cells stained lightly, and those of satellite cells moderately in the mouse (Fig. 17), rabbit,[8] guinea-pig,[3,11] man,[3] and rat.[3] The ground substance, composed of nerve fibers and their sheaths, stained in a diffuse granular fashion. With longer incubation periods there was light staining of the cytoplasm of ganglion cells on occasion. With group B substrates, findings were similar except that ground substance stained more deeply (mouse, Fig. 18).

Action of Inhibitors

Cyanide. (Fig. 52.) The staining of structures demonstrated with group A substrates was inhibited by incubation in the presence of M/100 KCN. Under conditions of maximum staining, staining of nuclei was less inhibited than that of cytoplasm, as for example in the proximal convoluted tubules of the kidney and in the endothelial cells of blood vessels. Staining of structures demonstrated with group B substrates, which also stained with group A substrates, was inhibited approximately to the same degree when incubated in the presence of M/100 KCN; structures staining exclusively with group B substrates, such as smooth muscle in some species, were inhibited to a lesser degree.

Glycine. (Figs. 42, 45, 48, 58, and 60.) The findings with M/4 glycine were similar to those with M/100 KCN for all substrates.

Arginine. (Figs. 43, 46, 50, and 53.) When arginine was used in concentrations of M/4, the findings resembled those with M/100 KCN and M/4 glycine, but the degree of inhibition was much less.

Bile Salts. (Figs. 62, 63, and 66.) With glycerophosphate and muscle adenylic acid as substrates, sodium taurocholate or glycocholate at a concentration of 0.006 M inhibited to a degree comparable with that of M/4 arginine.

Trichloroacetic Acid. (Fig. 56.) Dipping deparaffinized sections for 10 minutes in 5 per cent trichloroacetic acid prior to incubation inhibited the staining of all structures. The inhibition was almost complete at sites of group I and group II activity, and was considerably greater than that observed with M/100 KCN or M/4 glycine. The inhibiting effect was less upon the staining of nuclei (group III).

Heat. (Fig. 54.) Heating deparaffinized sections in distilled water at 80° C. for 10 minutes prior to incubation produced the same or even more extensive inhibition than did dipping in 5 per cent trichloroacetic acid.

Iodoacetic Acid, Sodium Azide, Sodium Fluoride, and Formaldehyde. Incubation in the presence of M/500 iodoacetic acid, M/500 sodium azide, and M/100 sodium fluoride, or dipping sections for 10 minutes in 4 per cent formaldehyde solution prior to incubation with group A and group B substrates produced variable results. On the average, there was little or no effect. Occasionally, there might be slight activation or inhibition and infrequently these were somewhat more marked, particularly the activation on dipping sections in 4 per cent formaldehyde for 10 minutes prior to incubation.

Action of Activators

Magnesium Ion and Ascorbic Acid. Incubation in M/100 to M/1000 magnesium salts or M/100 ascorbic acid increased the staining of all structures demonstrated with group A and group B substrates.

As has been repeatedly demonstrated chemically, incubation with low concentrations of the inhibitors such as M/100 glycine and M/100 arginine and M/2500 KCN resulted in activation of the enzymes. This has also been demonstrated histochemically. The salts of the bile acids in low concentrations, M/1000 sodium taurocholate, glycocholate, and desoxycholate, also were activators, and seemed to produce a more distinct increase in the staining of nuclei than of cytoplasm (Figs. 64 and 67).

The results with the various substances noted above were regularly reproducible. They depended, however, upon the concentration of enzyme in the various tissues and frequently residual staining was observed in those structures which showed the highest concentration of enzyme, although complete inhibition occurred at sites where enzyme concentration was low. The total amount of enzyme activity demonstrable in a given tissue depends upon the amount initially present and any loss or inactivation which may occur as a result of the fixing and embedding processes. Figures 45 and 46 show extensive inhibition in

mouse kidney, except for residual staining of the brush border and Figures 42 and 43 show complete inhibition in a section of mouse ovary on the same slide incubated in the presence of M/4 glycine and arginine as compared with the control sections of these organs (kidney, Fig. 44; ovary, Fig. 41).

Discussion

The histochemical technic for visualizing sites of alkaline phosphatase activity in fixed tissue sections[1-3] has come into widespread use. With the resolution of various objections,[31,52,53] there are few who would doubt that the method is fundamentally sound. As with all procedures, it must be used with adequate controls based upon sound judgment and for relatively limited purposes. For instance, the use of fixed tissue necessarily introduces all of the uncertainties of precise localization of the enzyme that are involved in the extension of any histologic data on fixed tissue to the original living cell. Since it has been clearly shown by several workers[31,54] that some inactivation occurs at almost every step in the preparation of paraffin sections, it is evident that the enzyme activity of the fixed tissue is but a fraction of that originally present. However, unless very minute amounts were present at certain sites, such that complete inactivation occurred, it would still be possible to establish the precise location of the enzymes in tissue cells by selecting a suitable incubation time. The method is so sensitive that even if we accept the percentage estimates of inactivation given by Danielli[31] and Stafford and Atkinson,[54] enough enzyme still remains for purposes of localization without necessitating unduly long incubation times. Fortunately, a single molecule of active enzyme is capable of hydrolyzing many substrate molecules so that it only becomes necessary to select a suitable incubation time to secure a desired intensity of deposition of calcium phosphate. For instance, one molecule of catalase has been calculated to decompose 5,000,000 molecules of hydrogen peroxide in 1 minute at 0° C.[27]

While it is doubtful whether the relative amounts of enzyme destroyed in fixation and embedding are the same in various laboratories, or even in different blocks of tissues prepared in the same laboratory, the general uniformity of distribution observed by different workers suggests that this variation does not create any difficulties.

Points of difference may arise from the various procedures used for visualizing the deposited calcium phosphate. Most laboratories have converted the calcium phosphate to cobalt sulfide. It has been the experience in this laboratory that control sections which have not

been exposed to substrate, but which were dipped into cobalt solution after having been in calcium nitrate, frequently showed a non-specific deposition of cobalt which subsequently appeared as cobalt sulfide. Gomori mentioned initially[1] that cobalt and nickel on long exposure form compounds with tissue proteins, and Krugelis[55] mentioned non-specific staining in control sections finished with cobalt. Accordingly, in this study the von Kossa technic has been employed exclusively for the demonstration of calcium phosphate.

The present observations require the assumption of three groups of phosphate-liberating enzyme systems with maximum activity at about pH 9.2. The first group is primarily cytoplasmic and occurs characteristically in the endothelial cells of capillaries and other small blood vessels in most organs (Figs. 41 and 51), in the proximal convoluted tubules of the kidney (Figs. 7 to 15), in the cortical cells of the adrenal gland (Figs. 47 and 49), and in other locations as described in detail above; it represents the cytoplasmic enzyme of previously reported histochemical investigations.[1,3,5,7,11,22] These group I enzymes are adequately demonstrated with all of the substrates, except that yeast nucleic acid (Fig. 33), thiaminepyrophosphate (Fig. 35), and adenosinetriphosphate (S) (Fig. 40) are least satisfactory because of the crystalline character of the precipitate and lack of precise delineation of structures. Enzymatic activity is quite high since it can be demonstrated with periods of incubation of 1 to 2 hours (Figs. 17, 41, and 44), and is completely or almost completely inhibited by incubation in the presence of M/4 glycine[5,7,56] (Figs. 42, 45, 48, 58, and 60), or arginine[56] (Figs. 43, 46, 50, and 53), M/100 KCN[44,57] (Fig. 52), 0.00625 M sodium taurocholate or sodium glycocholate[36] (Figs. 62, 63, and 66), or prior to incubation by dipping sections in 5 per cent trichloroacetic acid for 10 minutes (Fig. 56) or by heating at 80° C. for 10 minutes (Fig. 54). Group 1 enzymatic activity was relatively unaffected by incubation in the presence of M/100 NaF, M/500 iodoacetate or azide, and 0.00125 M sodium taurocholate, sodium glycocholate, or sodium desoxycholate; activation was observed by addition of M/100 Mg^{++},[5,56] ascorbic acid, glycine, or arginine[56] to the substrate mixture.

Group I enzymes correspond in their properties to the alkaline phosphatase in blood serum and are probably the predominant enzymes described by numerous investigators in extracts of various organs prepared by autolysis.[27] It was possible to confirm many earlier observations made chemically using organ extracts for group I enzymes, such as rates of hydrolysis, pH optimum, action of inhibitors, and

substrate specificity. Levene and Dillon,[58] using a phosphatase preparation obtained from intestinal fistulas in dogs, noted that nucleotides were hydrolyzed about as rapidly as sugar esters, while nucleic acids were split much more slowly. This is in agreement with the histochemical behavior of group I enzymes in the present study. The pH optimum for this group with all of the substrates used was at about pH 9.2 with a range extending from pH 8.6 to 9.6, which is in agreement with chemical studies.[27] Variations in intensity and incomplete staining were noted frequently on either side of the pH optimum. Bodansky[56,59] has established differences in the inhibiting power of amino acids for certain organ extracts, and in agreement with his observations a given molar concentration of glycine, M/4 to M/8, was more effective than the same concentration of arginine in inhibiting group I enzymes in tissue sections (Figs. 45 and 46).

It has not been possible to establish the presence of substrate specific group I enzymes with the phosphate esters used in the present study. Thus the sites of localization, pH optima, and reactions to inhibitors were the same regardless of substrate used, although the character and color of the precipitates varied as noted above. Data as to substrate specificity using organ extracts are somewhat divided. In a chemical study, Winnick[60] showed that creatine phosphate was hydrolyzed by alkaline phosphatases, and he was unable to support the claims of several earlier investigators[60] for the existence of a separate and specific creatine phosphatase. Gomori[61] has reported the preparation of a specific hexosediphosphatase, but this enzyme is inactivated by alcohol and acetone and hence would not be demonstrable in fixed tissues; Zorzoli and Stowell[42] were unable to demonstrate a specific hexosediphosphatase by histochemical means. In the present study, no evidence of a specific hexosediphosphatase was found. This is not to imply that substrate specific enzymes do not exist, but merely that they are not demonstrated using fixed tissues (Figs. 7 to 13, 21 to 25, and 30 to 36).

In connection with the localization of substrate specific enzymes by histochemical means, the objections raised[62] to the terminology used by Glick and Fischer[63-66] are pertinent. These workers employed adenosinetriphosphate as substrate and stated that they were demonstrating adenosinetriphosphatase in mouse heart and in wheat grain. It has been shown[67,68] that alkaline phosphatases hydrolyze adenosinetriphosphate, so that the assumption of a specific adenosinetriphosphatase was permissible only in those tissues which did not contain alkaline phosphatases. There is little doubt that a specific adenosine-

triphosphatase distinct from the enzyme splitting the 5-linkage (see group II below) does exist, but it is questionable whether it can be visualized histochemically in fixed tissues in view of the thermal lability of this enzyme.[69] The failure of magnesium to inactivate this enzyme[70] and the lack of activation by molar glycine[71] in tissue sections suggest that a specific adenosinetriphosphatase is not being demonstrated in the tissue sections.

The second group of enzymes is present characteristically in the muscular walls of the medium-size arteries (Figs. 2 and 4), in the muscular walls of some of the hollow viscera (Figs. 6, 18, 26, 27, 29, 37 to 40, and 59) in certain species, and in other locations as described in detail above. It is best demonstrated by the use of muscle adenylic acid and adenosinetriphosphate as substrates, in which the phosphate molecules are linked to the fifth carbon of the ribose portion of the molecule. It is not demonstrable to any degree with yeast adenylic acid (Figs. 24 and 34), in which the phosphate molecule is attached to the third carbon atom of the ribose, as substrate, nor is it demonstrated by the use of other substrates. Group II enzymes are differentiated from the group I enzymes by differences in location and by this relative substrate specificity. These differences may be noted with short periods of incubation of 1 to 4 hours (Figs. 17 and 18). With periods of incubation approaching 1 week, some precipitate is noted in these sites with the other substrates; this may possibly be attributable to a very slow rate of splitting of the other substrates.[72]

The enzymatic activity of this group is inhibited and activated by the same substances that inhibit group I enzymes, but it is noted that a concentration of inhibitor which will completely inhibit group I activity only partially inhibits group II enzymes. Thus, in a section of gut using sodium-beta-glycerophosphate as substrate and incubated in the presence of M/4 glycine, complete inactivation is noted (Fig. 58), while an adjacent section, using muscle adenylic acid as substrate in the presence of M/4 glycine, will show staining persisting at sites of group II activity, but somewhat decreased in intensity, although the mucosal staining due to the action of group I enzymes is completely inhibited (Fig. 60). The pH optimum of this group is at about pH 9.2 and extends over a range of pH from 8.6 to 9.6.

The outstanding characteristic of group II enzymes lies in their relative specificity for the 5-linkage. Species differences are particularly significant with respect to group II enzymes as noted in the detailed descriptions above.

Group II enzymes appear to correspond to the 5-nucleotidase ex-

tracted from tissues by Reis[28] in 1934 and by Gulland and Jackson.[72] This enzyme was found to be distributed through the tissues of several animal species, with the exception of skeletal muscle. These same tissues displayed negligible activity when yeast adenylic acid (adenosine-3-phosphoric acid) was used in place of muscle adenylic acid (adenosine-5-phosphoric acid).[28] Different samples of adenosinetriphosphate gave staining of varying intensity and character, although the sites of localization were the same (Figs. 39 and 40). These samples consisted of the calcium salt (A), the dibarium salt (Bs), and adenosinetriphosphate as the free acid (S). Bailey,[73] working with the barium salt, has noted that on standing, adenosinetriphosphate spontaneously hydrolyzes, liberating adenosinediphosphate and inorganic phosphate and also muscle adenylic acid and pyrophosphate. It is possible that the variations between the preparations were due to hydrolysis resulting in differences in their adenosinetriphosphate and muscle adenylic acid content.

The third group of enzymes is present in the nuclei of most cells. It is differentiated in part from the group I enzymes by differences in the rates of hydrolysis of the various substrates (Figs. 21 to 27, and 30 to 40). Muscle adenylic acid, yeast adenylic acid, creatine phosphate, and sodium-beta-glycerophosphate were split most rapidly; adenosinetriphosphate (Bs), glucose-1-phosphate, and hexosediphosphate were split at an intermediate rate; while adenosinetriphosphate (A), adenosinetriphosphate (S), nucleic acid, and thiaminepyrophosphate were split least rapidly by nuclei. The differences were small but consistent, and of significant degree. For example, with hexosediphosphate as substrate, the staining of tissues with group I activity in the cytoplasm is as dark or darker than similar staining in an adjacent section using creatine phosphate as substrate. However, the nuclei on the same section are invariably much better stained with the latter substrate (compare Figs. 32 and 36). Its separate identity from group I enzymes is further indicated by the action of M/100 KCN, M/4 glycine or arginine, and by dipping sections in 5 per cent trichloroacetic acid or by heating sections in water at 80° C. for 10 minutes. While these substances inhibit both types of enzymes, the inhibition of group I enzymes is much greater. In favorable instances, the group I enzymes can be almost completely inhibited, so that group III nuclear enzymes are observed almost exclusively (Figs. 48, 50, 52 to 54, and 56). The nuclear enzymes are also differentiated from the group II cytoplasmic enzymes by differences in substrate specificity. Thus creatine phosphate is split by the group III nuclear en-

zymes more rapidly than adenosinetriphosphate (Bs), but is split slightly or not at all by group II enzymes. In addition group III enzymes were found to be inhibited by salts of bile acids in 0.00625 M concentration (Figs. 62, 63, and 66); and were unaffected by M/100 NaF and M/500 iodoacetate and azide, and activated by M/100 Mg^{++}, ascorbic acid, glycine and arginine, and 0.00125 M sodium taurocholate, sodium glycocholate, and sodium desoxycholate (Figs. 64 and 67).

Group III is further characterized by a comparatively slow rate of splitting of substrates as compared with the other two groups, so that at best, longer periods of incubation approaching 24 hours are necessary for its best demonstration. In studies of group III enzymes in the presence of inhibitors, even longer periods of incubation, up to 1 week, are required.

The pH optimum of this group of enzymes also was found at about pH 9.2 but good staining was noted from pH 8.0 to 9.4. More consistent nuclear staining was seen toward the acid side, although the absolute maximum of intensity was usually at about pH 9.2.[35] Attempts to separate nuclear from cytoplasmic phosphatases utilizing pH differences alone have not proved successful. In the present study, although all nuclear enzymes have been grouped together, it is not improbable that the nuclei of cells showing cytoplasmic group I enzymes may contain some of this enzyme in addition to the group III enzymes. A similar situation may also hold for nuclei of cells with cytoplasmic group II enzymes. There is some suggestive evidence that more than one nuclear enzyme may exist in other nuclei. For instance, in adjacent sections of guinea-pig intestine, using glycerophosphate and muscle adenylic acid as substrates and periods of incubation of 24 hours, fibrocytic nuclei in the submucosa which were unstained using glycerophosphate as substrate, were stained when muscle adenylic acid was used as substrate and persisted after incubation in M/4 glycine, while fibrocytic nuclei in the lamina propria and nuclei of the mucosa which stained with glycerophosphate were completely inhibited with this concentration of glycine in both sections (Figs. 57 to 60). As has been mentioned, autolysates of rat intestine or guinea-pig kidney and adrenal gland prepared according to Bodansky[36] contain a dialysable substance,[37] which is unaffected by boiling for 30 minutes and which on addition to the substrate mixture greatly enhances nuclear staining so that after only 2 hours of incubation, nuclear staining is readily discernible even in tissues which ordinarily show negligible nuclear staining in this interval. Activation of group I and II cytoplasmic staining also occurs. Ek, von Euler, and Hahn,[37] in a chemical

study of phosphatases prepared from calf intestinal mucosa, found that reactivation of dialyzed phosphatase could be accomplished by addition of either boiled phosphatase solution or of alanine, and suggested that the latter might be the activating factor.[37] Fischer and Greep[74] found that commercial trypsin, crystalline trypsin, and crystalline bovine serum albumin all had an activating effect on purified intestinal phosphatase[68] as did amino acids or amino acids plus inorganic ions such as magnesium. These observations are in accord with the histochemical findings. Further studies to ascertain the chemical nature of the activating material are in progress.

Some observations on the effects of inhibitors on all three groups of enzymes are of interest. It was observed that those tissues which showed the highest concentrations of enzyme were the least affected by a given concentration of inhibitor (Figs. 41 to 46). For instance, the group I enzymes in the brush border of the proximal convoluted tubule and in the adrenal cortex showed residual enzymatic activity when incubated in the presence of M/100 KCN, M/4 glycine, and arginine, although all other sites of group I activity were completely inhibited; higher concentrations of inhibitor eliminated this residual staining.

It is recognized that the separation into three groups may not correspond with any of the usual biochemical classifications in that similar histologic localizations were found with substrates of great biochemical diversity. This might well be due to the presence of complexes of several enzyme systems at various sites of which only the terminal enzyme would actually cause the liberation of phosphate. For instance, if the usual alkaline phosphatase were unable to split glucose-1-phosphate directly, the combined presence of phosphoglucomutase which would transform the glucose-1-phosphate to glucose-6-phosphate, and alkaline phosphatase which liberates phosphate from the glucose-6-phosphate, would produce a histologic picture corresponding to that with glycerophosphate. Similar considerations may also obtain with respect to other substrates.

Chemical and histochemical findings are not concordant with respect to the salts of the bile acids. Bodansky found[36] by chemical assay that bile salts decreased considerably the action of bone and kidney phosphatases without affecting intestinal phosphatases. In his studies with 0.00625 M sodium taurocholate, bone phosphatase showed 53 per cent of its original rate of hydrolysis while intestinal phosphatase showed no significant reduction of hydrolysis of sodium-beta-glycerophosphate. Histochemical studies under the same conditions showed

that 0.00625 M sodium taurocholate and sodium glycocholate produced considerable inhibition of all three groups of enzymes in both kidney and intestine of man, rat, mouse, and rabbit (Figs. 62 and 63), whereas 0.00125 M sodium taurocholate, sodium glycocholate, and sodium desoxycholate had no inhibitory effect and produced activation in many instances (Fig. 64). No explanation can as yet be proposed for the discrepancy between chemical and histochemical data on the action of the salts of bile acids. It is noted that Emmel[44] found that the inhibitory effect of KCN on mouse kidney phosphatase is completely reversible when the KCN is washed out of the section prior to incubation in substrate, while the effect on mouse intestinal phosphatase is only slightly reversed under the same circumstances. No evidence for such differences has been obtained in the present study using various substrates and inhibitors, although reversibility of inhibiting effects has not been studied.

The differentiation of these groups of enzymes serves to explain in part some of the differences noted when alkaline phosphatases are studied using various substrates. Dempsey, Deane, Wislocki, *et al.*[22,38–41,50,75] believed that they had seen differences in localization when glycerophosphate, glucose-1-phosphate, yeast adenylic acid, hexosediphosphate, yeast nucleic acid, and lecithin were used as substrates. These were interpreted as indicating that a considerable number of substrate specific enzymes were involved. Our data with the first five of these substrates when used in equimolar phosphate concentration do not support this view (Figs. 7 to 13, 21 to 25, and 30 to 36). The nature of the differences stated to exist by Dempsey *et al.*[22,38–41,50,75] has been difficult to evaluate, and indeed they frequently intimate that the different substrates give essentially similar pictures. Minor differences described by these workers can probably be explained as a result of the differences in the rates of hydrolysis of the various substrates by the three groups of enzymes delineated above, and by the variations noted away from the pH optima. The present observations only indicate substrate specificity in group II enzymes and these are specific for the ribose-5-phosphate linkage, hitherto unreported by histochemical technics (Figs. 2, 4, 6, 18, 26, 27, 29, 37 to 40, and 59).

Suggestions as to the separability of the phosphatases in terms of nucleus and cytoplasm, either stated or implied, are to be found in earlier histochemical papers by Bourne,[11] Danielli and Catcheside,[20] Lorch,[30] Krugelis,[55] and Morse and Greep.[35] It is evident that the use of histochemical technics allows for the separation of the phosphatases into nuclear and cytoplasmic components. At the present time, chemi-

cal studies of the differences between nuclear and cytoplasmic phosphatases would be very difficult to carry out. Histochemical methods also permit the simultaneous study of the action of inhibitors, pH, and substrate specificity among various organs of different species in a much shorter time than would otherwise be possible, and such investigations may well contribute to an understanding of the rôle that the phosphatases play in various metabolic phenomena and in pathologic states.[5,6,12,15,17,18,49,51,76–80]

Summary

The histochemical technic for localizing alkaline phosphatases in tissues has been investigated using a variety of substrates and of substances which inhibit or enhance enzymatic activity.

Three mutually separable groups of phosphatases acting at about pH 9.2 have been recognized by considering substrate specificity and rate of hydrolysis, effect of inhibitors and activators, and differences in sites of occurrence.

One of these groups of enzymes (group II) corresponds to 5-nucleotidase previously reported from chemical studies but not previously demonstrated histochemically.

Detailed observations of the histochemical localization of these various groups of enzymes in human, rabbit, guinea-pig, rat, and mouse tissues have been made.

An activator of all three groups of phosphatases has been shown to occur in dialysates of autolyzed rat intestine and of guinea-pig kidney and adrenal gland. It is not magnesium or glycine.

Certain technical difficulties usually encountered in histochemical studies have been considered. In particular, non-specific deposition of calcium phosphate on the slide has been shown to be due to action of enzyme in solution which has probably been leached out of the tissue sections.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

All sections were counterstained with eosin. In no instance was a nuclear stain used. In most cases adjacent serial sections were photographed, but occasionally, *e.g.*, Figures 1 and 2, closely adjacent but not serial sections were used.

Photomicrographs at a particular magnification were taken under identical photographic conditions, with a green filter (Wratten B no. 58) and Eastman Kodak Panatomic X film. Magnifications were as follows: Figures 1 to 56, × 120; Figures 57 to 60, × 60; Figures 61 to 67, × 240. Within any series of photomicrographs, the exposure and development of negatives and prints were identical, so that variations in intensity are due to differences in the character of the precipitates when different substrates were used.

PLATE 43

Rabbit heart, coronary artery; pH 9.2, 1 week's incubation.

FIG. 1. Sodium-beta-glycerophosphate.

FIG. 2. Adenosinetriphosphate (Bs).

Guinea-pig heart, coronary artery; pH 9.0, 24 hours' incubation.

FIG. 3. Sodium-beta-glycerophosphate.

FIG. 4. Adenosinetriphosphate (Bs).

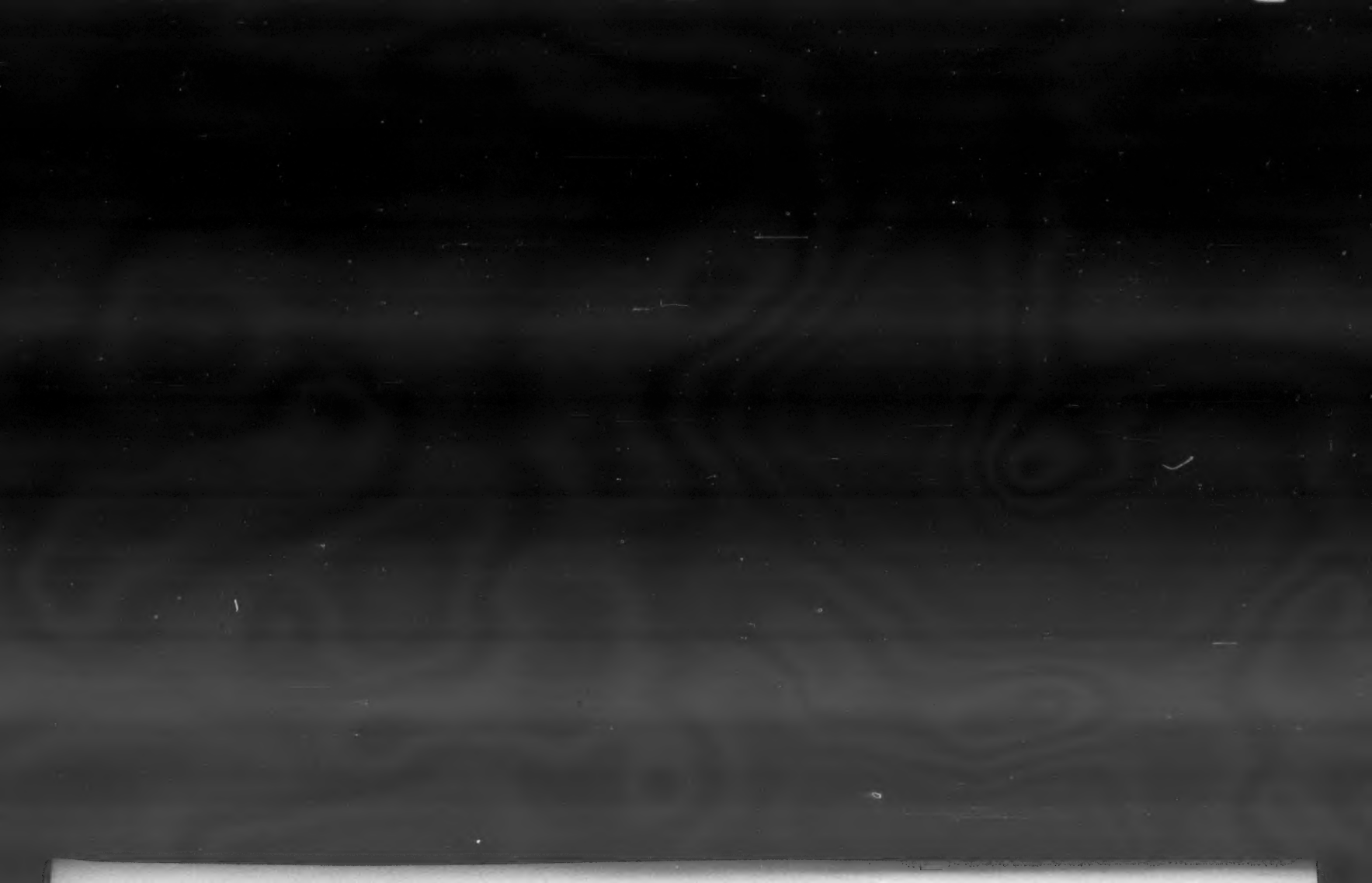
Sections stained with group B substrates (Figs. 2 and 4) show staining of media of coronary vessels which is not present with group A substrates (Figs. 1 and 3), indicating presence of group II enzyme. The myocardium shows essentially similar staining with all substrates.

Guinea-pig, small intestine; pH 9.2, 24 hours' incubation.

FIG. 5. Sodium-beta-glycerophosphate.

FIG. 6. Muscle adenylic acid (K).

Sections stained with group B substrates (Fig. 6) show intense staining in the muscularis and muscularis mucosae, indicating the presence of group II enzyme, not seen when group A substrates are used (Fig. 5). No essential differences in staining of other structures are evident. In Figure 5, capillaries and nerve plexuses stand out sharply against the relatively unstained muscularis.



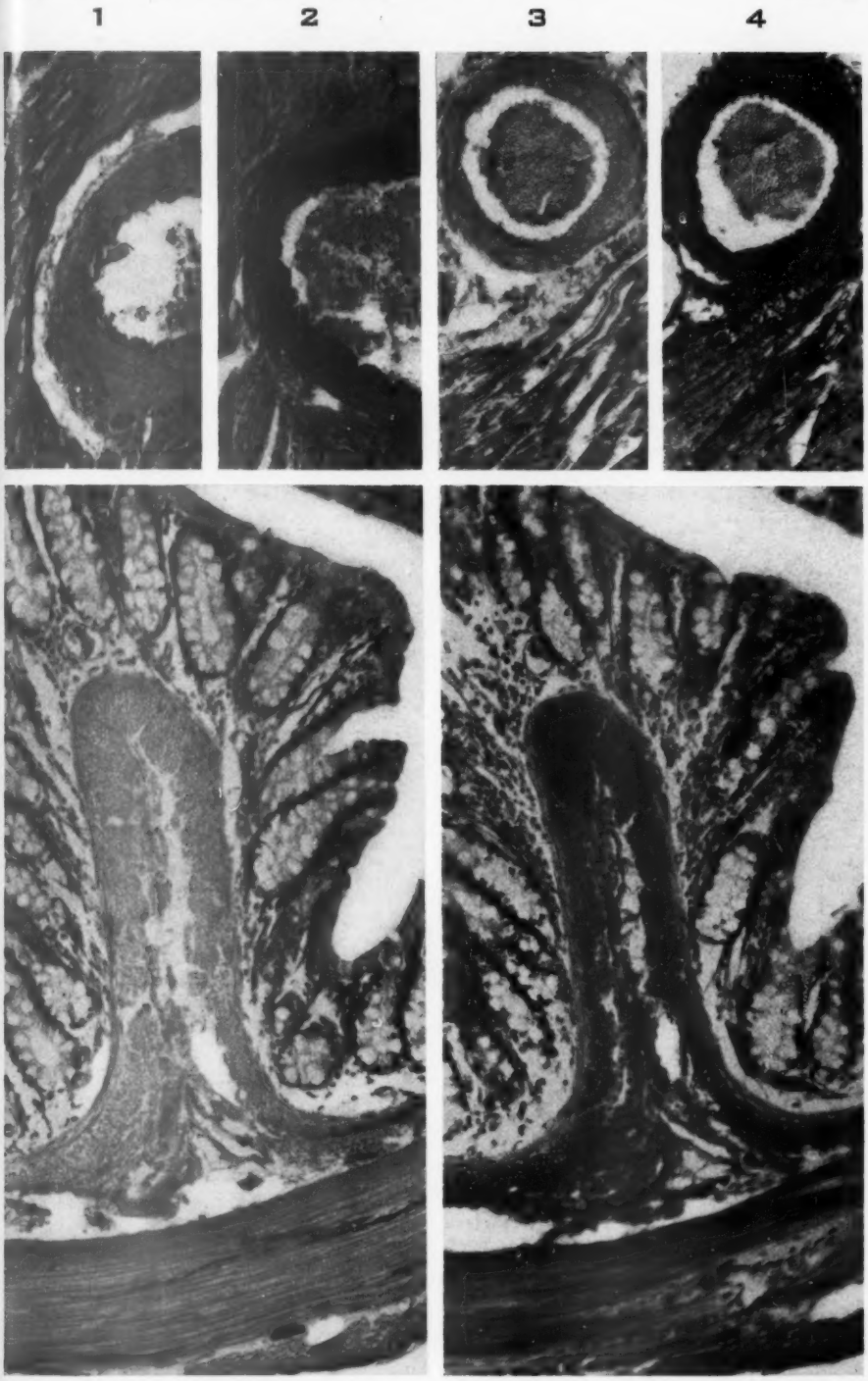

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Plate 44

Guinea-pig kidney, cortex; pH 9.2, 24 hours' incubation.

Fig. 7. Sodium-beta-glycerophosphate.
Fig. 8. Glucose-1-phosphate.
Fig. 9. Creatine phosphate.
Fig. 10. Yeast nucleic acid.
Fig. 11. Yeast adenylic acid.
Fig. 12. Thiaminepyrophosphate.
Fig. 13. Hexosediphosphate.
Fig. 14. Muscle adenylic acid (K).
Fig. 15. Adenosinetriphosphate (Bs).
Fig. 16. Control, no substrate.

In Figures 7 to 15, the sites of enzymatic activity are the same with all of the substrates. The differences in appearance are due to the variations in the character of the precipitate and to the intensity of enzymatic staining which is a function of rates of hydrolysis of the various substrates; the control section (Fig. 16) shows no enzymatic activity, but only the effects of the eosin counterstain.

Mouse ureter, pH 9.2, 2 hours' incubation.

Fig. 17. Sodium-beta-glycerophosphate.
Fig. 18. Muscle adenylic acid (K).

With group B substrates (Fig. 18), the ureteral muscle stains intensely, indicating site of group II activity, since this tissue is unstained with group A substrates (Fig. 17). The ureteral epithelium and the adventitia of the blood vessels stain similarly with both substrates. The adventitial staining of larger blood vessels is characteristic for the mouse and rat. The intensity of the staining of the ground substance of the autonomic ganglion with group B substrates, indicating site of group II activity, is greater.

Fig. 19. Mouse seminal vesicle; pH 9.2, 24 hours' incubation in sodium-beta-glycerophosphate.
Fig. 20. Mouse epididymis; pH 9.2, 24 hours' incubation in sodium-beta-glycerophosphate. The difference in depth of staining of the cytoplasm in adjacent areas of the epididymis was constant in the mouse and rabbit. Note the difference between the lightly stained cytoplasm in the upper portion of the illustration as contrasted with the intense staining of the cytoplasm in the lower portion.

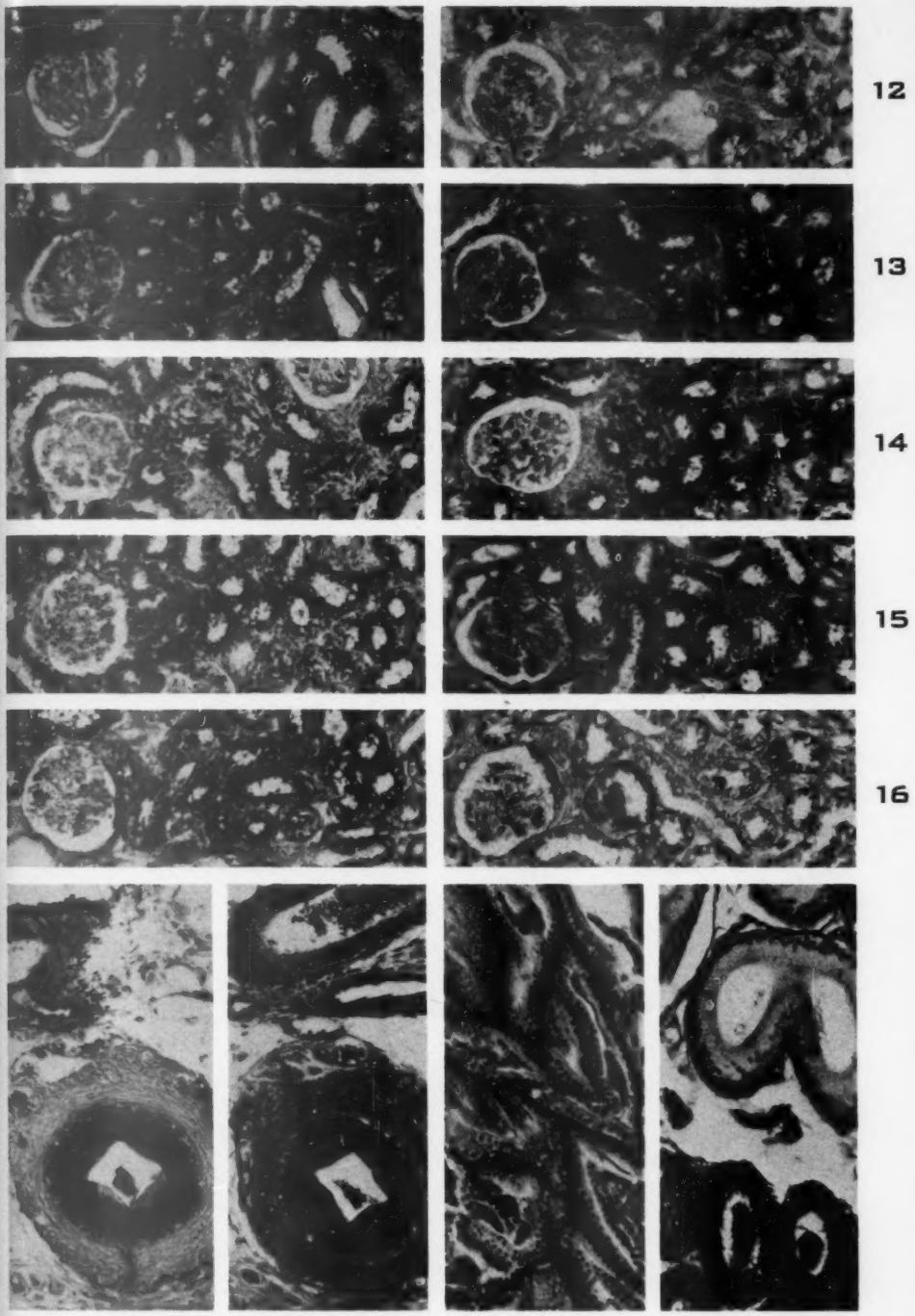
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PLATE 45

Guinea-pig urinary bladder; pH 9.2, 24 hours' incubation.

FIG. 21. Sodium-beta-glycerophosphate.
FIG. 22. Glucose-1-phosphate.
FIG. 23. Creatine phosphate.
FIG. 24. Yeast adenylic acid.
FIG. 25. Hexosediphosphate.
FIG. 26. Muscle adenylic acid (K).
FIG. 27. Adenosinetriphosphate (Bs).

With group B substrates (Figs. 26 and 27), intense staining of the muscularis, not seen with group A substrates (Figs. 21 to 25), is noted, indicating site of group II enzyme activity. Aside from this difference, localization of enzyme activity with all substrates (Figs. 21 to 27) is essentially similar. Better nuclear staining is seen in Figures 21, 23, 24, and 26, as compared with Figures 22, 25, and 27, indicating differences between group I and III enzymes.

Guinea-pig seminal vesicle; pH 9.2, 24 hours' incubation.

FIG. 28. Hexosediphosphate.
FIG. 29. Muscle adenylic acid (K).

With group B substrates (Fig. 29), intense staining of the muscularis is noted, not seen with group A substrates (Fig. 28), indicating site of group II activity.

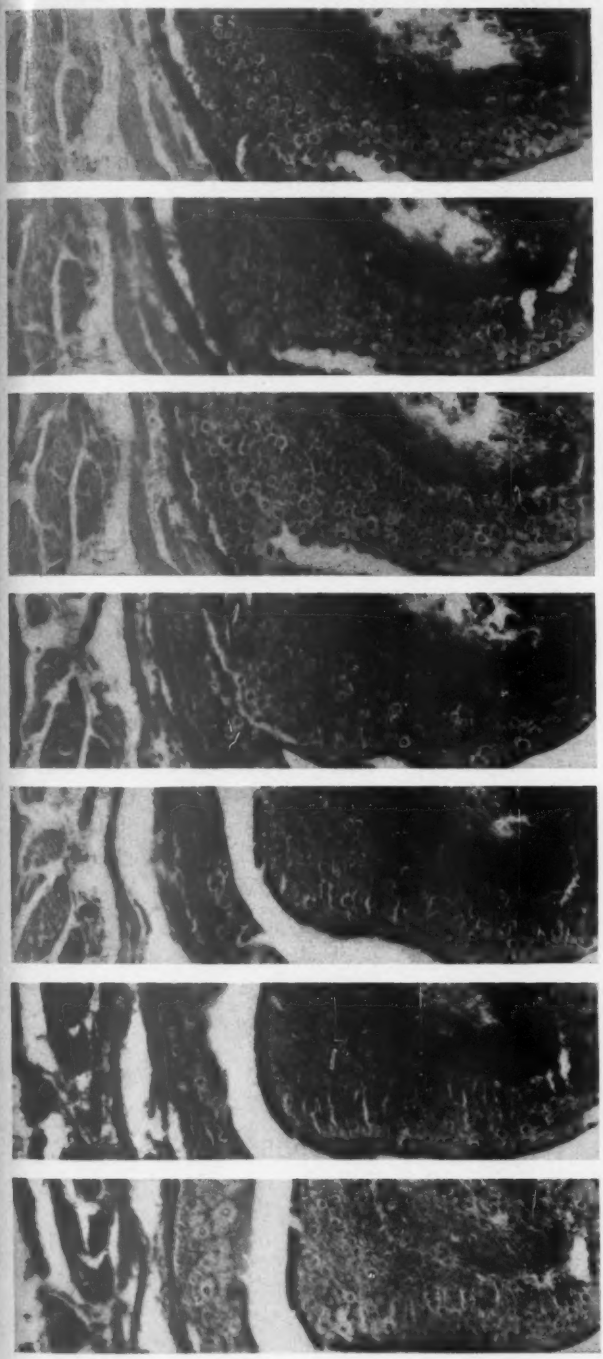

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PLATE 46

Rat uterus; pH 9.2, 24 hours' incubation.

FIG. 30. Sodium-beta-glycerophosphate.
FIG. 31. Glucose-1-phosphate.
FIG. 32. Creatine phosphate.
FIG. 33. Yeast nucleic acid.
FIG. 34. Yeast adenylic acid.
FIG. 35. Thiaminepyrophosphate.
FIG. 36. Hexosediphosphate.
FIG. 37. Muscle adenylic acid (K).
FIG. 38. Muscle adenylic acid (Bf).
FIG. 39. Adenosinetriphosphate (Bs).
FIG. 40. Adenosinetriphosphate (S).

With group B substrates (Figs. 37 to 40), there is intense staining of the myometrium, unstained with group A substrates (Figs. 30 to 36), indicating the site of group II activity. Superiority of nuclear staining is evident in Figures 30, 32, 34, 37, and 38, as compared with that in Figures 31, 36, and 39, aiding in the differentiation of group I and group III enzymes; cytoplasmic staining in all is approximately equal. Staining with yeast nucleic acid (Fig. 33), thiaminepyrophosphate (Fig. 35), and adenosinetriphosphate (S) (Fig. 40) is relatively poor, although in each instance the localization is characteristic of the respective substrate group.

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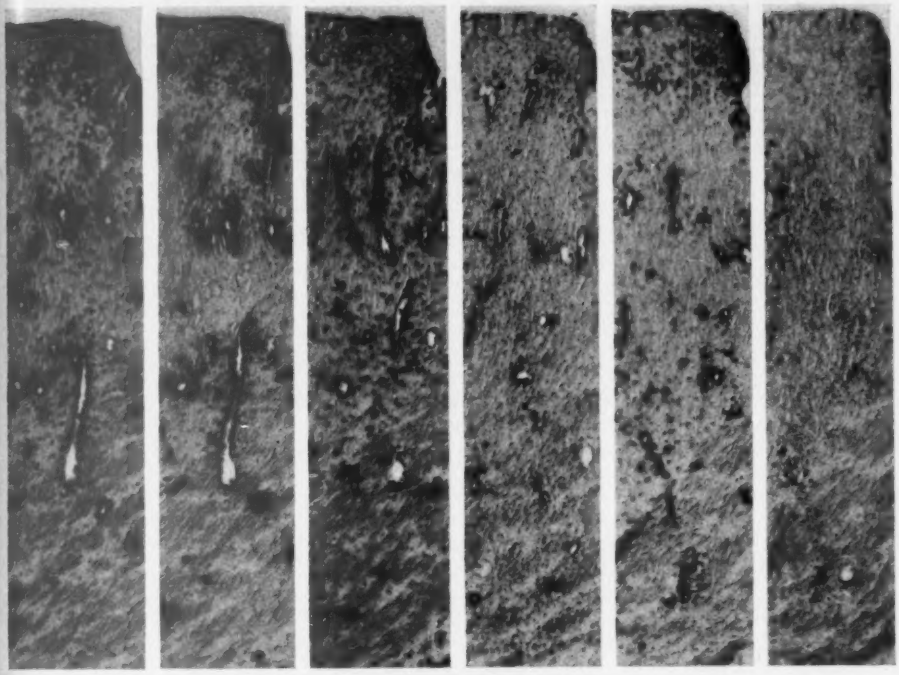

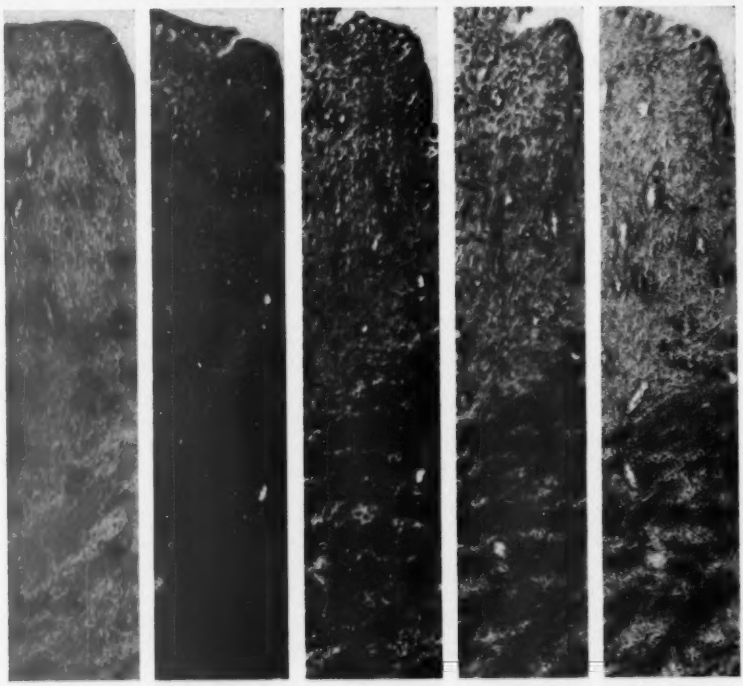

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Plate 47

Figs. 41 to 43, mouse ovary, and Figs. 44 to 46, mouse kidney; pH 9.2, 1 hour's incubation in sodium-beta-glycerophosphate.

Figs. 41 and 44. On same slide, no amino acid.

Figs. 42 and 45. On same slide, incubated in M/4 glycine.

Figs. 43 and 46. On same slide, incubated in M/4 arginine.

In two different organs on the same slide, the organ showing the greater enzyme activity, *i.e.*, kidney (Fig. 44), still shows staining with this concentration of amino acid (Figs. 45 and 46), while the section of ovary on the same slide (Fig. 41) shows complete inhibition of enzyme activity under these conditions (Figs. 42 and 43). M/4 glycine inhibits more than does an equivalent concentration of arginine (compare Figs. 45 and 46).

Figs. 47 and 48. Guinea-pig adrenal gland; pH 9.2, 24 hours' incubation in sodium-beta-glycerophosphate. Section illustrated in Figure 48 was incubated in the presence of M/4 glycine.

Figs. 49 and 50. Guinea-pig adrenal gland; pH 9.2, 1 week's incubation in sodium-beta-glycerophosphate. Section illustrated in Figure 50 was incubated in the presence of M/4 arginine.

The sections demonstrate that glycine and arginine in this concentration produce much greater inhibition of cytoplasmic group I enzymatic activity than of group III nuclear activity.

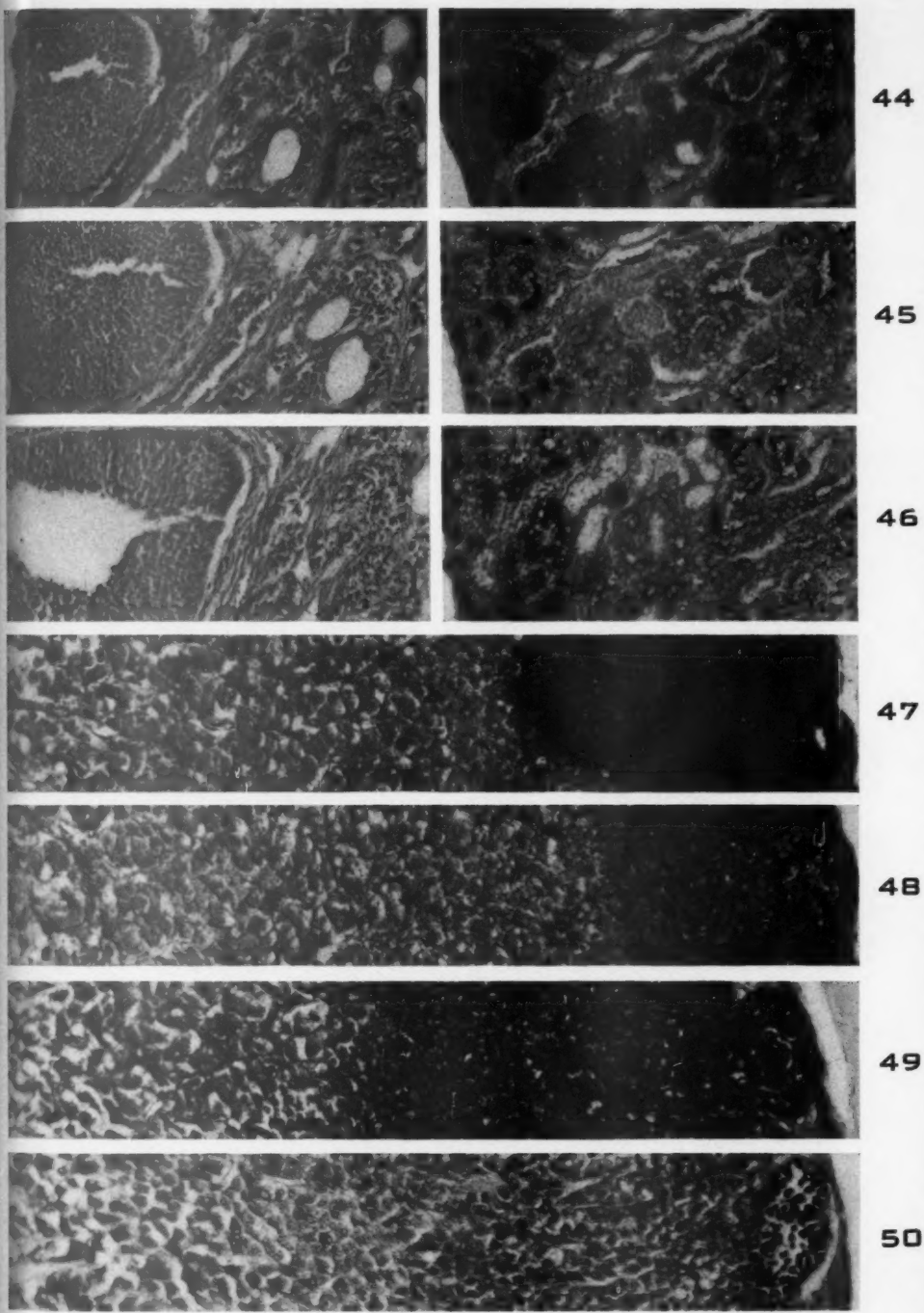
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PLATE 48

FIGS. 51 to 54. Human brain; pH 9.2, 1 week's incubation in creatine phosphate. Section illustrated in Figure 52 was incubated in the presence of M/100 KCN, that in Figure 53 in M/4 arginine, and that in Figure 54 was heated in distilled water for 10 minutes at 80° C., prior to incubation in the same substrate solution as the section in Figure 51. Cytoplasmic staining of small blood vessels and of the external glial membrane (group I) seen in Figure 51 has been inhibited by KCN (Fig. 52), arginine (Fig. 53), and heat (Fig. 54), while nuclear staining (group III) is relatively unaffected.

FIGS. 55 and 56. Guinea-pig renal cortex; pH 9.2, 1 week's incubation in creatine phosphate. Section in Figure 56 was in 5 per cent trichloroacetic acid for 10 minutes prior to being placed into the same substrate solution as used for the section illustrated in Figure 55. Trichloroacetic acid inhibits group I cytoplasmic activity while having relatively little effect on group III nuclear activity.

Figs. 57 to 60. Guinea-pig, small intestine; pH 9.2, 24 hours' incubation.

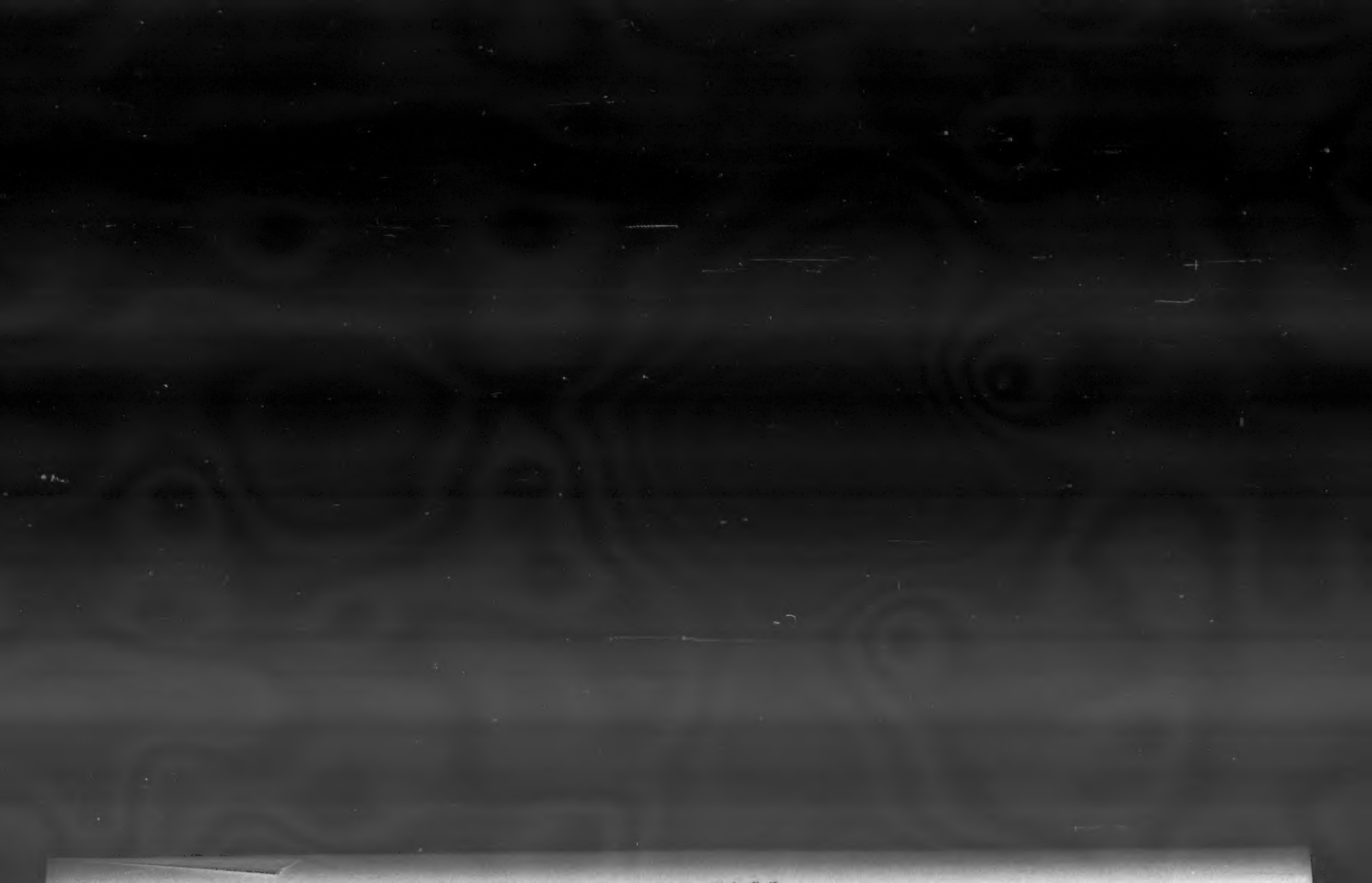
FIG. 57. Sodium-beta-glycerophosphate.

FIG. 58. Sodium-beta-glycerophosphate + M/4 glycine.

FIG. 59. Muscle adenylic acid (K).

FIG. 60. Muscle adenylic acid (K) + M/4 glycine.

Group II enzyme activity, illustrated by staining of the muscularis mucosae and muscularis with the group B substrates (Fig. 59), is inhibited to a lesser degree by glycine (compare Figs. 59 and 60), than is the group I enzymatic activity in the epithelium and lamina propria, which shows inhibition with both groups of substrates when incubated in the presence of M/4 glycine (compare Figs. 58 and 60).



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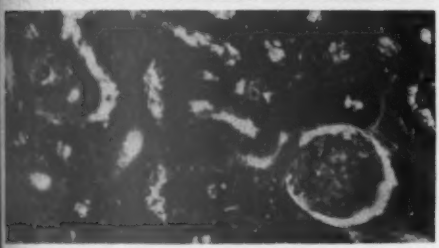

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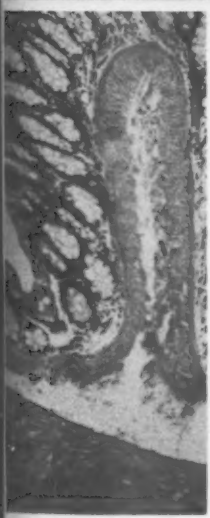

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PLATE 49

Rabbit intestine; pH 9.2, 4 hours' incubation in sodium-beta-glycerophosphate.

FIG. 61. No bile salt.

FIG. 62. 0.00625 M sodium glycocholate.

FIG. 63. 0.00625 M sodium taurocholate.

FIG. 64. 0.00125 M sodium desoxycholate.

There is a marked inhibitory effect of 0.006 M salts of bile acids in contrast to activating effect, especially on nuclei, of 0.001 M salts of bile acids.

Human liver; pH 9.2, 4 hours' incubation in sodium-beta-glycerophosphate.

FIG. 65. No bile salt.

FIG. 66. 0.00625 M sodium taurocholate.

FIG. 67. 0.00125 M sodium desoxycholate.

Definite inhibition with 0.006 M salt of bile acid (Fig. 66), and striking nuclear activation with 0.001 M salts of bile acids are seen. Figures 64 and 67 with 0.00125 M sodium desoxycholate are similar to those with the same concentrations of sodium glycocholate and sodium taurocholate.

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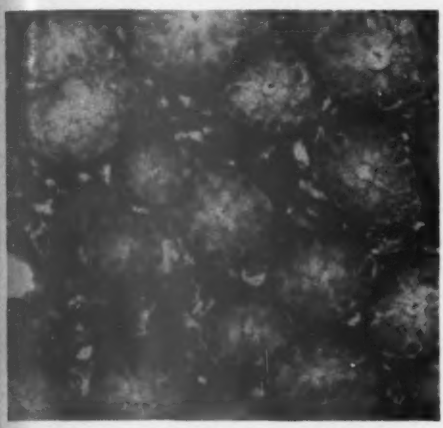

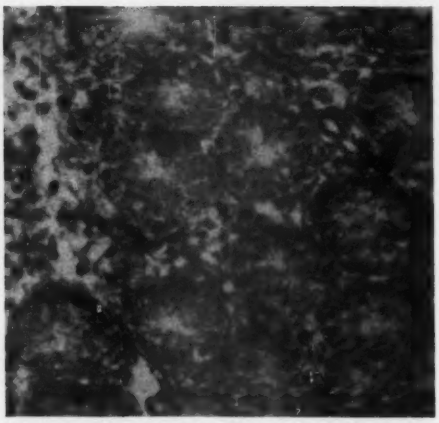

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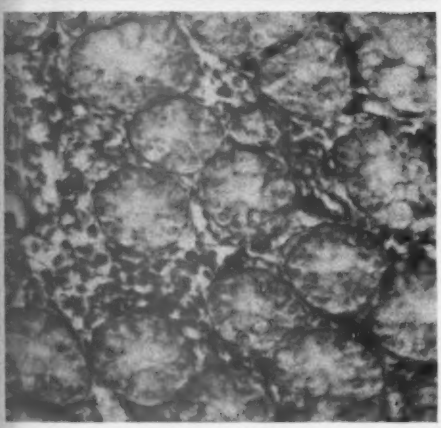

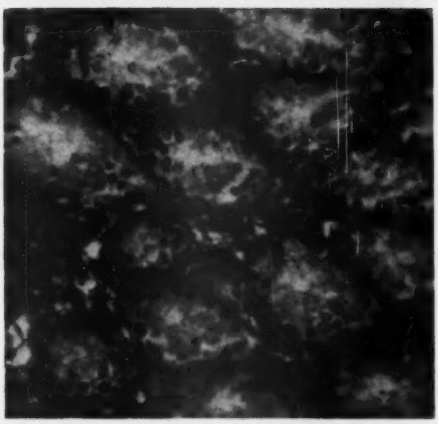

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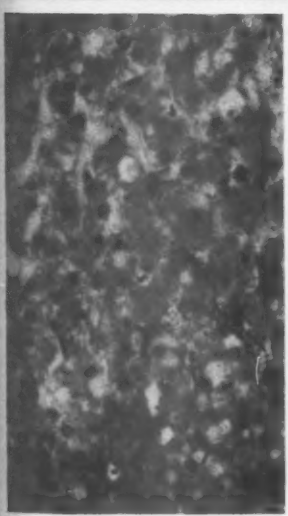

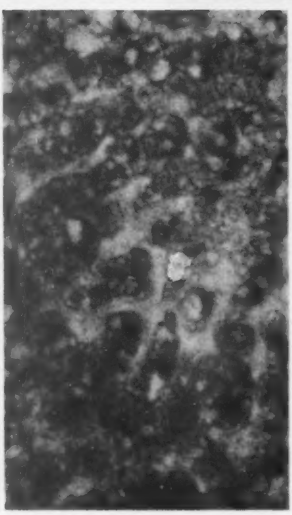

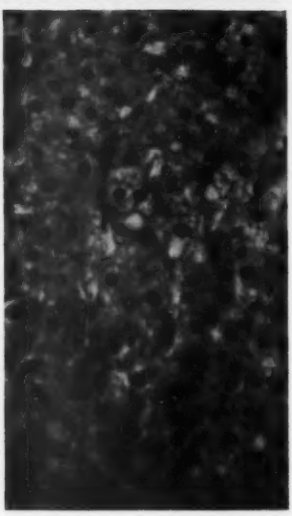

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THE EFFECT OF THE CALCIUM AND PHOSPHORUS CONTENT OF THE DIET UPON THE FORMATION AND STRUCTURE OF BONE*

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This paper presents a study of the blood, bone ash, and undecalcified sections of the bones of rats receiving adequate and optimum amounts of dietary calcium and various disproportions of calcium and phosphorus created by introducing a great excess of calcium in an otherwise normal diet. The experiments, which began in 1937 when the literature on the subject of calcium and phosphorus metabolism gained a high state of maturity, were stimulated by numerous related papers and monographs which appeared in the ensuing 10 years. To avoid unnecessary repetition and to minimize the space given to corroborative findings, we will present only the correlated observations of chemical, radiographic, and histophysiologic methods. The emphasis will be upon filling in the gaps in the literature which are due to the inaccuracy of data obtained from decalcified histologic sections. From the use of histologic methods not involving decalcification, similar to those described in previous studies from this laboratory,[1-4] we will present objective definitions for such terms, phrases, and words as osteoporosis, halisteresis, rickets, demineralization, decalcification and rarefaction of bone, increased or decreased density of bone, and osteomalacia, which are frequently used with broad meaning or upon an ill-established hypothesis.

To gain harmony with the literature, the present experiments were done with diets which have been standardized by well known studies in normal and pathologic nutrition. Sherman[5] and his co-workers investigated the calcium requirements of the laboratory rat and determined the composition of adequate and optimum diets. An *adequate* diet is a diet which will support normal growth, health, reproduction, and lactation, generation after generation; an *optimum* diet is a diet which will produce "increased growth, earlier maturity, higher adult vitality as indicated by superior breeding records, a longer period between the attainment of maturity and the onset of senility, and, in less

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degree, an increase in the average length of adult life." The bone structure of rats reared on these diets will be described in detail.

Shohl[6] reviewed his own work and the writings of many others who have established the fundamental facts about the diet-blood-bone relationships with respect to calcium and phosphorus. Brown, Shohl, Chapman, Rose, and Sauerwein[7] were among the first to emphasize the point that the interrelation of dietary minerals is as important as the actual amount of any single element. They found that a diet which permitted normal deposition of bone mineral was transformed into a rickets-producing diet by increasing the calcium while maintaining the phosphorus constant. They expressed these relationships in terms of the Ca/P ratio, and showed that diets with a ratio above 8 will be rachitogenic. We have repeated these experiments using similar diets of Ca/P ratios ranging from 0.5 to 8.6 in order to supply further information which can be gained from the study of undecalcified sections of the bones.

Materials

One hundred and forty-two Wistar-Sprague Dawley mixed strain stock rats were weaned at 21 days, divided into seven groups, and placed on various diets for 2, 3, 4, or 8 weeks.

Adequate Diets. Forty rats were placed on Sherman's diets nos. 16, 16A,[8] or on Bill's diet,[9] all of which contain a 1 to 1 or a 1 to 2 proportion of calcium to phosphorus in amounts of approximately 0.20 to 0.42 per cent calcium and 0.40 to 0.43 per cent phosphorus.

Optimum Diets. Forty-eight rats were fed Sherman's diets nos. 168,[10] 268,[11] or fox chow (purina), which contain calcium and phosphorus in proportions of 1 to 1 to 2 to 1 in amounts of approximately 0.64 to 1.44 per cent calcium and 0.40 to 1.06 per cent phosphorus.

High Calcium Diets. Nineteen rats were reared on a diet of the same foodstuffs as Sherman's diet no. 268 but containing calcium and phosphorus in proportions of 5 to 1 in amounts of approximately 2.0 per cent calcium and 0.43 per cent phosphorus. This and the diets listed below should be regarded as artificial diets because the proportion of calcium to phosphorus is more than 2 to 1 and much higher than ever occurs in natural foods.

High Calcium Rachitogenic Diets. Eleven rats were divided into two groups and placed on the following diets for 3 weeks: (1) Bill's diet, plus 6.0 per cent $CaCO_3$, producing a proportion of 6.7 to 1 calcium to phosphorus in amounts of approximately 2.8 per cent calcium and 0.42 per cent phosphorus. (2) Bill's diet, plus 8 per cent $CaCO_3$, producing a proportion of 8.6 to 1 calcium to phosphorus in amounts of approximately 3.6 per cent calcium and 0.42 per cent phosphorus.

High Calcium, Phosphorus-fortified Diet. Five rats were reared for 3 weeks on a diet of fox chow, plus 8.0 per cent $CaCO_3$, producing a 4.4 to 1 proportion of calcium to phosphorus in amounts of approximately 4.64 per cent calcium and 1.06 per cent phosphorus.

Methods

Body Weight. The rats were weighed twice weekly to determine the weight curves on each diet.

Body Length. The length of the animal from the tip of the nose to the tip of the tail was measured twice weekly to determine the over-all rate of growth.

Food Consumption. Food and water were administered *ad libitum*. The volume of food consumed by animals on each of these diets was estimated to determine the possible difference in the total over-all intake as well as that of calcium and phosphorus.

Serum Calcium and Phosphorus. Serum calcium was determined on the pooled sera of the animals in each group by Clark and Collip's modification of the method of Tisdall.[12] Serum inorganic phosphorus was measured by the method of Gomori.[13]

Serum Phosphatase. Alkaline phosphatase was measured on the pooled sera of each group by the method of Bodansky.[14]

Percentage of Ash. The percentage of ash was determined on the dried, fat-free, left femur of each rat. The length and density of the metaphyses of the individual animals were examined roentgenologically.

Histologic Studies. The left tibiae were sectioned without decalcification and stained with silver, hematoxylin, and eosin by the methods of McLean and Bloom.[1] For finer cellular detail, the opposite tibiae were fixed in Zenker's fluid-formaldehyde solution and sectioned after decalcification. By means of undecalcified sections, the presence of osteoid tissue, calcified bone with osteoid borders, or fully calcified bone trabeculae was determined. Osteoporosis was estimated by both the number of bone trabeculae found across the spongiosa, and by whether or not the shaft contained areas of rarefaction. The appearance and number of layers of osteoblasts and the size and number of osteoclasts in the spongiosa were noted. The thickness of the epiphyseal plate and the length of the spongiosa were measured with a micrometer.

Results

Although the animals in all of these experiments were autopsied and examined, for the sake of brevity only those observations are presented which have a direct bearing upon the subject of this paper.

Diets of Adequate and Optimum Calcium Intake

A summary of the experimental data obtained from animals on so-called normal diets is shown in Table I (Fig. 1). A comparison of the bone changes and other differences between animals on Sherman's adequate and optimum diets is listed in Table II (Figs. 2 to 4). These findings represent the picture which was seen in the average rat rather than in the most abnormal or the most nearly normal experimental animal. Wide individual variations may be expected in all experiments dealing with diets fed *ad libitum* and influenced by seasonal factors in vitamin D metabolism.

It should be emphasized that the bone changes were most extreme in animals at 7 weeks of age, after 4 weeks on the diets. The differences in the effects of the diet upon bones, as observed both histologically and by percentage determinations of ash, were diminished rather than accentuated by a longer period of deprivation. The most likely explanation for this, as will be discussed further, is that morphologic changes are accentuated in young animals; the demands of the body for calcium are greatest between 3 and 8 weeks of age, which is the period of most rapid growth. Furthermore, it is to be noted that the principal difference in the animals on optimum diets was in the earlier development of the normal bone structure of the adult.

High Calcium Diets

Rats reared on diets containing 1.2 per cent more calcium than is contained in Sherman's optimum diet showed inhibitory effects upon growth, but both advantageous and adverse changes in the bones (Fig. 1). The animals increased in weight more slowly than animals on adequate diets, and after 8 weeks on the diet averaged approximately 5 per cent less than the rats on adequate or optimum diets. The nose-to-tail measurements showed that the animals were approximately the same size as animals on adequate diets, but 3 per cent smaller than animals on optimum diets. These differences may be due, in part, to the fact that the volume of food consumed by these rats was slightly less than that of rats on adequate and optimum diets. The serum calcium tended to be 1 to 2 mg. per cent higher and the serum phosphorus 1 mg. per cent lower during the first 4 weeks on the diet. The serum alkaline phosphatase rose steadily and reached a peak at 4 weeks at 43 mg. per cent, and was always higher than in animals on either adequate or optimum diets. The percentage of ash of the skeleton was approximately the same as that found in animals on adequate diets. Roentgenograms of the skeleton showed the metaphyses to be both longer and denser than in animals on adequate diets, but 0.75 mm. or so

TABLE I

The Growth, Blood, and Bone Changes in Rats on "Normal" Diets of Varying Calcium and Phosphorus Content

Period and diet	Increase in weight over period of experiment		Increase in length over period of experiment		Ash	Blood		Spongiosa length		Epiphyseal plate thickness	
	Males	Females	Males	Females		Ca	P	Tibia	Femur	Tibia	Femur
	%	%	%	%	%	mg. %	mg. %	mm.	mm.	mm.	mm.
Two weeks:											
1. Sherman's diet 16[8]	77.10	84.60	28.35	31.35	45.37	10.80		1.22	1.30	0.359	0.331
2. Sherman's diet 268[11]	90.90	104.50	33.18	34.60	50.47	9.30		1.61	2.08	0.351	0.292
3. High Ca diet (2%)	70.20	71.00	30.06	31.10	45.60	10.00	10.20	1.53	1.47	0.497	0.385
Three weeks:											
1. Sherman's diet 16	168.10	137.80	35.28	33.60	42.04	11.30		1.40	1.57	0.342	0.399
2. Sherman's diet 268	172.00	172.40	37.70	39.45	49.98	11.20		2.56	2.91	0.337	0.300
3. High Ca diet (2%)	107.80	110.10	34.12	32.80	43.94	12.00		2.31	2.14	0.351	0.366
Four weeks:											
1. Sherman's diet 16	180.30	165.30	50.00	50.00	48.43	8.95	10.20	1.09	1.66	0.246	0.239
2. Sherman's diet 268	239.20	203.80	59.80	59.20	49.10	11.00	10.30	2.62	2.48	0.292	0.323
3. High Ca diet (2%)	154.20	116.10	49.60	37.00	48.10	12.10	7.30	2.14	2.93	0.376	0.311
Eight weeks:											
1. Sherman's diet 16	463.00	288.00	92.80	72.70	52.24	9.94	6.90	2.17	2.08	0.217	0.187
2. Sherman's diet 268	407.00	348.10	86.60	77.90	55.26	10.14	8.13	1.84	2.07	0.212	0.177
3. High Ca diet (2%)	404.00	245.00	80.25	72.70	52.38	10.67	8.07	2.51	2.70	0.258	0.225

shorter and somewhat less dense than in animals on optimum diets (Table II).

Microscopic examination of the undecalcified bones showed an epiphyseal cartilage which was slightly wider than in animals on adequate or optimum diets. The zone of provisional calcification was 3 hypertrophic cells in depth and the new bone trabeculae were laid down at intervals of 3 or 4 rows of cartilage cells, rather than 5 to 7 cells as on adequate diets or 2 or 3 cells on optimum diets. This resulted in a spongiosa more dense than in animals on adequate diets but less dense than in rats on optimum diets. The spongiosa was slightly longer than in animals on adequate diets and the trabeculae were generally fully calcified and without osteoid borders. The shaft contained areas of fibrous connective tissue and vascular structures in the same proportions as those found in rats on adequate diets. Osteoblast and osteoclast counts also were much the same as in animals on adequate diets.

TABLE II

Comparative Analysis of Young Rats after Four Weeks on Diets Containing Adequate and Optimum Amounts of Calcium

Observation upon	Adequate diet	Optimum diet
Weight gain	71 to 89 gm.	75 to 95 gm.
Increase in tail to nose length*	10.3 cm.	11.1 cm.
Average serum calcium	10.0 mg. %	Same as on adequate diet
Average serum phosphorus	6.0 to 10 mg. %	Same as on adequate diet
Alkaline phosphatase†	24 to 28 units	Same as on adequate diet
Percentage of ash	42 to 52%	49 to 55%
Length of the metaphysis in roentgenograms	0.5 mm.	1.5 mm.
Density of the metaphysis in roentgenograms	25% of that of the cortex	75% of that of the cortex
Thickness of the epiphyseal cartilage	0.25 mm.	0.21 mm.
Depth of the zone of provisional calcification	3 hypertrophic cartilage cells	5 or 6 hypertrophic cartilage cells
Intervals of cartilage cell rows between bone trabeculae	5 to 7	3 to 4
Length of the center of the primary spongiosa	1.1 mm.	1.5 mm.
Osteoid borders	On bone everywhere	None
Porosity of the shaft	Extensive	Relatively slight
Osteoblasts	Very abundant, everywhere	Relatively less abundant
Osteoclasts	Numerous	Relatively few

* In 4 weeks.

† Milligrams of phosphate split off in 1 hour.

At 11 weeks of age, after 8 weeks on the diets, the roentgenographic and histologic differences between the rats on adequate, optimum, and high calcium-content diets almost completely disappeared from the bones of most of the animals.

High Calcium Rachitogenic Diets

Rats reared on diets which were normal with respect to phosphorus (0.4 per cent) and vitamin D, but containing more than 3 per cent calcium, developed rickets similar to animals on diets containing 2.0 to 3.9 per cent strontium,[15] beryllium,[16] iron,[17] magnesium,[18,19] lead,[20] or thallium carbonate[21] (Fig. 5). The resulting condition may be termed "high-calcium rickets." In young rats reared for 3 weeks on the Bill's supplemented high calcium diets listed in Table III the serum calcium

TABLE III

Blood and Bone Changes in Young Rats on Normal Diets with Excessive Supplements of Calcium

Composition of diet	Diet		No. of animals	Serum		Spongiosa length		Epiphyseal plate thickness		Weight at autopsy	Rickets
	Ca	P		Ca	P	Tibia	Femur	Tibia	Femur		
	%	%		mg. %	mg. %	mm.	mm.	mm.	mm.	gm.	
Bill's diet[9] plus 6% $CaCO_3$	2.80	0.42	5			2.09	1.83	0.37	0.45	60	Minimal or moderate
Bill's diet plus 8% $CaCO_3$	3.60	0.42	9	13.38	7.56	1.54	1.43	0.39	0.40	50	Severe
Fox chow checkers plus 8% $CaCO_3$	4.64	1.06	5	12.12	8.68	2.46	2.10	0.35	0.38	76	Minimal or none

was 1 mg. per cent higher and the serum inorganic phosphorus was within normal range or lower than normal. The epiphyseal plate was thicker than normal and a typical rachitic metaphysis was formed. All the bone trabeculae contained wide osteoid borders and the shaft was considerably rarefied. There was irregular or complete failure of calcification in the zone of provisional calcification of the cartilage matrix, which condition by definition is pathognomonic of rickets. Most of the above changes were prevented when the animals were fed a basic diet (fox chow) which provided as much or more phosphorus than the added calcium could withdraw in the intestinal tract (Fig. 6).

DISCUSSION

The changes in the skeleton of animals on calcium-deficient diets are obvious enough to be seen by gross observations of the growing ends of the bones. This was the only method by which Bauer, Aub, and Albright[22] observed that the metaphyses of the long bones can be almost completely absorbed in young cats placed on a calcium-deficient

fish diet. A modern method of measuring the bone calcium stores is by ash determinations of the skeleton or the whole body. Although ash determinations give excellent quantitative insight into the amount of mineral deposition, Shohl[23] was one of the first to appreciate that the method "obviously cannot differentiate between rickets and osteoporosis, between failure to ossify and demineralization." He studied the skeletal changes in animals on diets of varying calcium and phosphorus content by means of comparing the ash content with the histologic changes found in decalcified sections of bones of the same animal. Wolbach[23] examined the sections independently, and found that the rachitic metaphysis developed in rats on a high calcium-low phosphorus, low calcium-high phosphorus, or a low calcium-low phosphorus diet. Shohl and Wolbach[23] suggested that the Ca/P ratio of the diet indicated its rachitogenic properties, but also noted that in the low calcium-low phosphorus diet the Ca/P ratio was the same as that of various normal diets.

Bergeim,[24] Kramer and Howland,[25] and recently Holtz, Popper, and Silbermann,[26] who measured the urinary and fecal phosphorus excretion and determined the calcium balances of animals on various diets, have shown conclusively that the absorption of calcium is greatly influenced by chemical reactions with phosphorus in the gut. When large supplements of calcium were fed to rats on a normal diet, the fecal phosphorus excretion increased greatly and the absolute calcium and phosphorus content of the animal body gradually decreased. The diet changes from a rachitogenic to a non-rachitogenic diet when greater amounts of phosphorus are added.[7,23] Evidently, many other factors are introduced when the phosphorus content of the diet is increased. Such factors as caloric intake and the pH of the diet also are important in determining rachitogenic properties.[27] High phosphate diets when neutral in residue do not produce rickets. It is also to be noted that the rats on diets containing high calcium supplements showed signs of malnutrition and inanition in the later stages of these experiments. In some instances, starvation may have prevented the development of rickets.

Sherman's experiments[5] dealt with relatively neutral diets and, as demonstrated above, required undecalcified histologic preparations to show all of the differences that were present in the skeleton of animals on so-called "adequate" and "optimum" levels of calcium intake. Sherman observed that the rats on diets providing only limited or barely adequate amounts of calcium for normal life showed a relatively lower rate of skeletal and over-all body growth. The presence of a greater

supply of calcium was the incentive for the formation of large deposits of metaphyseal bone. The foregoing observations add that the optimum diet, having a Ca × P product of 0.32, showed metaphyseal bone stores with a denser and longer spongiosa, and heavier bone trabeculae which were completely calcified. The so-called adequate diet, on the other hand, showed a short spongiosa, a relatively rarefied metaphysis, and trabeculae which were incompletely calcified. In the strict sense of the term, these animals also had minimal rickets, disclosed by the consistent failure of the new bone to calcify promptly. However, reminiscent of the early description of rickets in wild rats captured in the laboratory by Erdheim,[28] rats on optimum diets may also not be the "normal."

The morphologic and chemical changes in the bones were appreciable in the foregoing experiments *only* during the short period between 3 and 8 weeks of age, or during the period when growth was most rapid and the extra-skeletal as well as the skeletal demands for calcium were very high. At 11 weeks of age, when growth was not yet complete but only somewhat diminished in rate, the skeleton received more calcium for storage and many of the structural differences disappeared from the bones of animals on adequate and optimum diets. It is important to recognize, therefore, that morphologic changes in the skeleton were brought out clearly only in young animals, and that the same changes and, no doubt, their reversal would require a much longer period of time and probably even a very great deprivation. Similar to the foregoing experiments, the testimony for the "lability of bone," frequently seen in the literature, is based upon observations on immature rather than adult animals with a relatively stable skeleton.

Aside from the effects of the dietary calcium and phosphorus upon the mechanism of calcification and the process of storage of bone salt, there remain other more obscure effects upon the growth of both soft parts and skeleton and the formation of bone matrix. Diets low in calcium, such as Sherman's "adequate" diet, did not promote growth as well as the optimum or high calcium diets, and produced a moderate form of rickets. In general, the skeletons of rats on limited amounts of calcium contain less osseous tissue than is found in rats on high calcium diets. The skeleton of such calcium-deprived animals may be described as severely osteoporotic and slightly rachitic. The latter term is used only because there was an irregular failure of calcification along the epiphyseal junction. It seems from these findings that the normal concentration of calcium ion in the body fluids is necessary for the growth of all of the tissues, including bone, whether or not other conditions are present which are necessary for calcification.

Definitions

The literature concerning the physiology and pathology of bone is confused by the use of terms to which different meanings are attached by different authors, or to describe observations made by different technics, *e.g.*, roentgenograms and histologic sections. This section attempts to give precise meaning to such terms as are commonly used to describe bone. The terms which have been used in this paper are used in the sense of the definitions which follow:

Decreased density of bone is a phrase used frequently by roentgenologists and indicates diminished volume of calcified bone tissue. Large amounts of osteoid may be present as in osteomalacia and rickets which, if calcified, would restore the normal density in roentgenologic examinations. In animals on so-called adequate calcium intake, the bone structure in roentgenograms was less dense than is normal, similar to that of younger rats but due to both insufficient bone formation and incomplete calcification of the bone trabeculae.

Demineralization of bone is a poor expression sometimes used by pathologists and radiologists to describe osteoporosis. Demineralization of bone tissue or "decalcification of the skeleton" does not occur in the sense that bone mineral is removed apart from bone tissue.

Halisteresis is a term denoting removal of bone salt from the bone tissue, leaving uncalcified bone matrix. This term should be discarded because bone salt has been observed to be mobilized *in vivo* only by one process in which the substrate or bone matrix disintegrates and dissolves at the same time that the inorganic material is released. We have observed mobilization of the bone salt in this way in hyperparathyroidism, vitamin D overdosage, and recovery from estrogen treatment. It may be assumed that the calcium salt can be dissolved out of the bone without destruction of the bone matrix *in vitro* only at a pH which probably cannot be developed under physiologic conditions. Evidence is available from studies with radioactive isotopes which indicates that ion exchange takes place between the body fluids and bone mineral. The bone matrix does not seem to lose inorganic mineral by this process, but may change its composition qualitatively.

Increased density of bone is a phrase often employed by radiologists, which should be used to designate a relative increase in the number of bone trabeculae in the spongiosa and a relative increase in the amount of calcified tissue in the cortex. These observations account for the relatively high ash content and the greater density of the roentgenograms of the bones of rats of all ages on Sherman's optimum diets.

Osteoid designates bone tissue formed but uncalcified, usually seen

under conditions which create a deficiency of calcium or phosphate ion in the blood and tissue fluids. Osteoid was found in rats on diets which provided only limited quantities of calcium and were described in the literature as "adequate." Osteoid was found also in rats on diets containing a great excess of calcium which withdrew phosphorus in an insoluble non-absorbable form in the gastro-intestinal tract.

Osteomalacia is a condition of the mature skeleton in which the new bone fails to calcify promptly and osteoid tissue is laid down upon previously formed bone tissue everywhere in the shaft and spongiosa. This was the condition of the bones of animals reared on a limited supply of calcium or on diets which withdrew phosphorus in the gastro-intestinal tract after growth was nearly completed.

Osteoporosis is a condition in which the bones are more porous than normal owing to (1) insufficient new bone formation to keep pace with the rate of bone resorption and thus preserve the equilibrium which retains the normal physiologic density of the skeleton, or to (2) under-development which accounts for the large areas of vascular and connective tissue in the shafts of the bones of growing rats on diets containing limited quantities of calcium.

Rarefaction of bone is synonymous with osteoporosis and decreased density of bone.

Rickets designates a condition of the immature skeleton in which there is (1) failure of provisional calcification in the epiphyseal lines during endochondral ossification, (2) delay in removal of cartilage, (3) osteoid borders formed around all of the bone trabeculae.

Summary

The condition of the bone-calcium deposits was observed and the turnover of the body calcium stores was estimated from undecalcified histologic sections of the upper tibiae of rats on diets of various composition with respect to calcium and phosphorus.

The bones of animals on various diets classified in the literature as "adequate" show: (a) a relatively short metaphysis and therefore a relatively short supply of stored calcium, (b) great osteoblastic and osteoclastic activity which reflects the need for maximum turnover and utilization of the bone calcium, (c) osteoid borders on the trabeculae indicating that calcium salt is not being stored to the maximum capacity of the bone structure, (d) a porous shaft similar to that of very young animals.

The bones of rats on various diets classified by Sherman as "optimum" for growth and longevity show: (a) a relatively long and dense

metaphysis which constitutes the maximum storage of bone salt, (b) diminished osteoblastic and osteoclastic activity which indicates minimum turnover and maximum conservation of calcium salt, (c) bone trabeculae calcified to the maximum density with no osteoid borders, which fact is evidence of an abundant and continuous supply of calcium and phosphorus in the diet and body fluids, (d) a very dense shaft similar to that of adults.

The bones of animals on diets in which the calcium intake alone is increased without increasing the phosphorus, show the deleterious results of this practice. Depending upon the absolute amounts of calcium added, the phosphorus is withdrawn from the diet in the intestinal tract in insoluble form and various changes occur in the bone in the following sequence: (a) incomplete calcification of the new bone as seen by osteoid borders of the trabeculae, (b) the zone of provisional calcification in the epiphyseal cartilage diminishes in depth or disappears completely, (c) invasion and removal of epiphyseal cartilage are retarded, resulting in the formation of a wide epiphyseal plate and a typical rachitic metaphysis.

On the basis of the above and other observations, various terms commonly used in describing the condition of the bones have been defined on an objective rather than a hypothetical basis.

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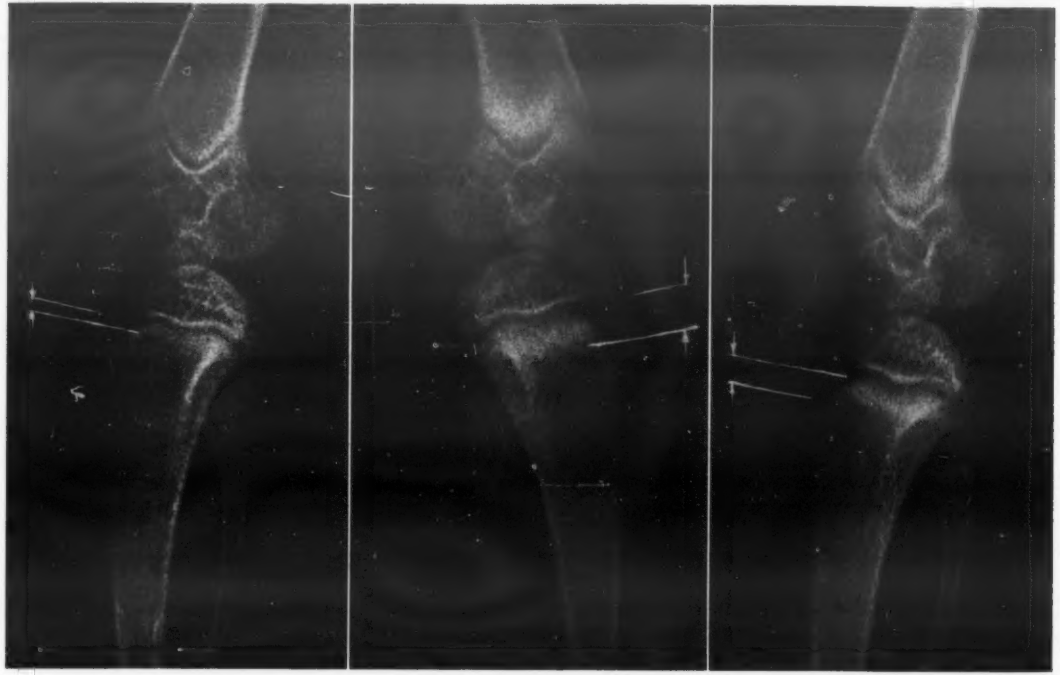
[*Illustrations follow*]

DESCRIPTION OF PLATES

PLATE 50

FIG. 1. Roentgenograms of the upper end of the tibia of littermate rats, 7 weeks of age, after 4 weeks on the following diets: A, adequate calcium, 0.20 per cent; B, optimum calcium, 0.80 per cent; C, high calcium, 2.0 per cent. The differences in the length of the spongiosa are shown by the distance between the lines and arrowheads. Of note also are the differences in the thickness of the cortex.



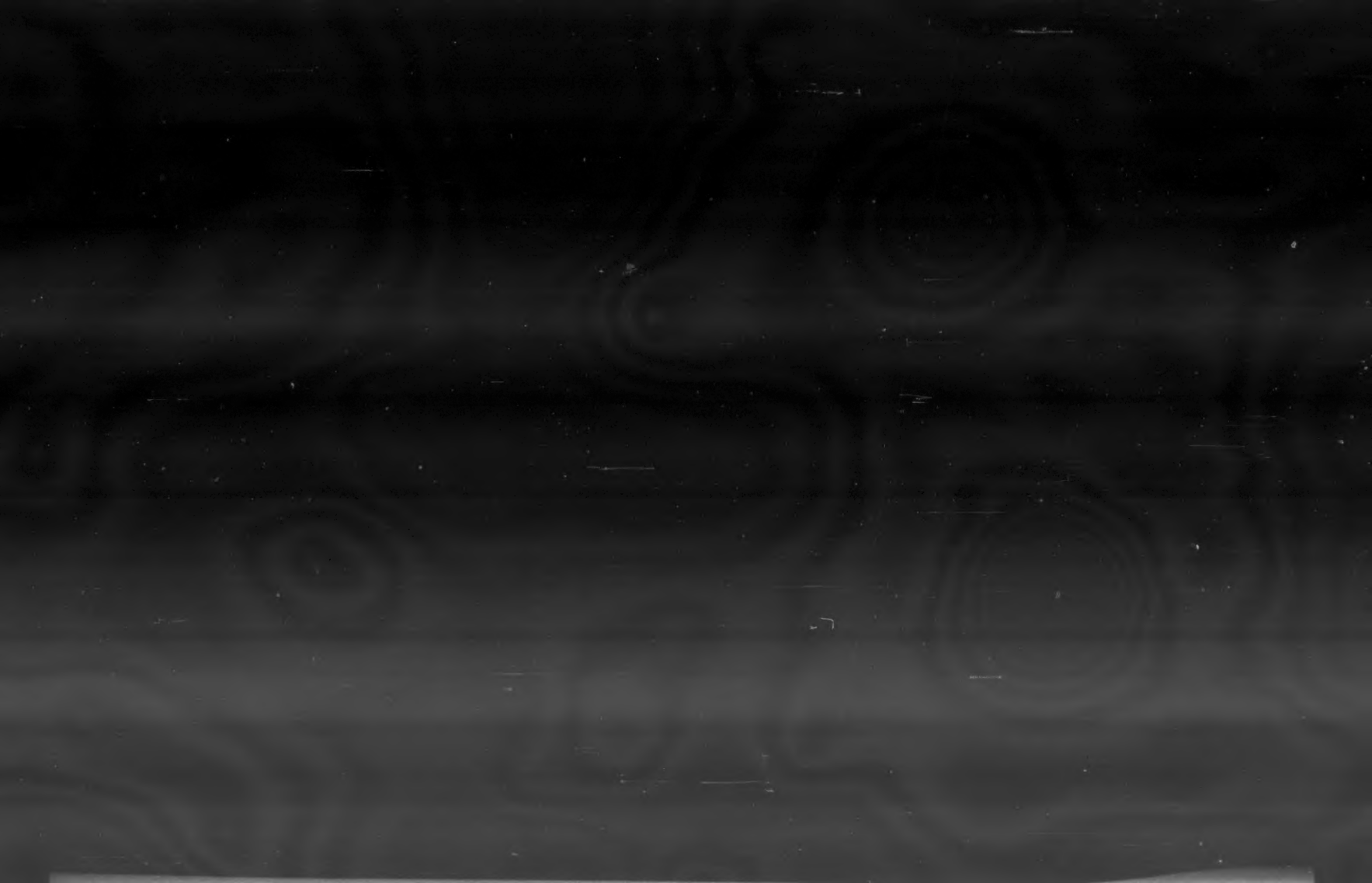
A B C

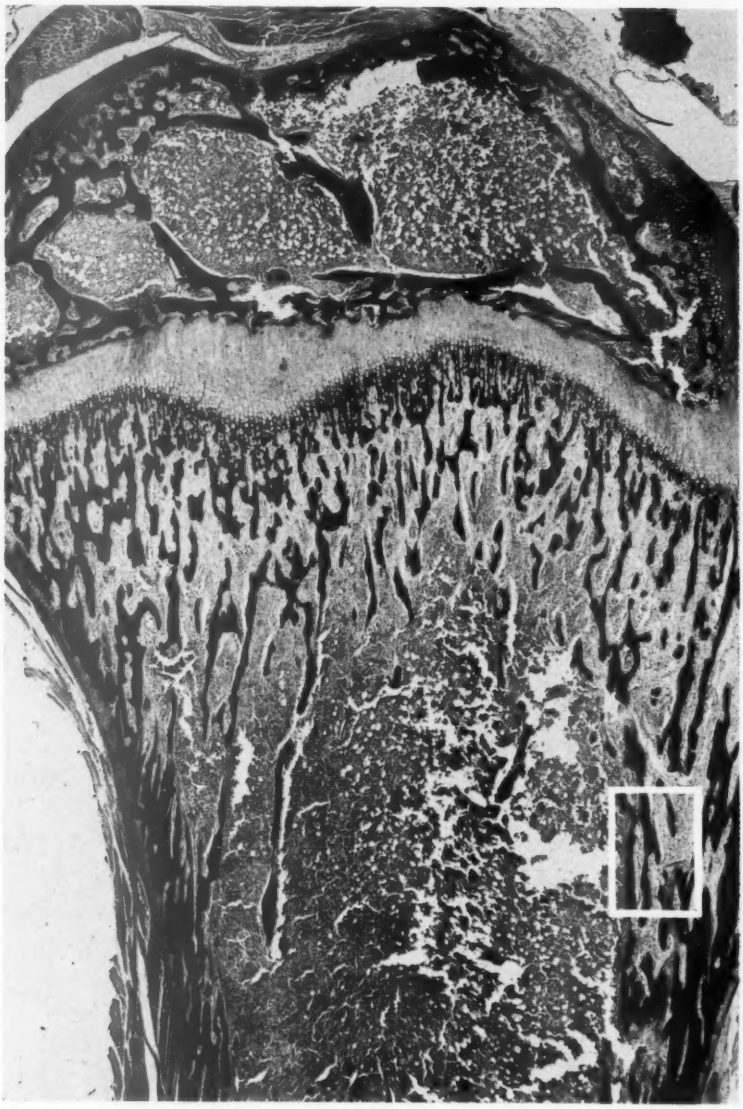
Carttar, McLean, and Urist

Dietary Calcium and Phosphorus

PLATE 51

FIG. 2. The upper end of the tibia of a rat reared on a so-called adequate diet, during the period of 3 to 7 weeks of age. Attention is called to the thickness of the epiphyseal cartilage and the depth of the zone of provisional calcification of the cartilage matrix; the thickness and number of trabeculae in the metaphysis; the density of the shaft. All of these may be compared with the same features in Figure 3, which is an enlargement of the area within the rectangle shown in Figure 2. From an undecalcified section stained in silver-hematoxylin and eosin. × 25.



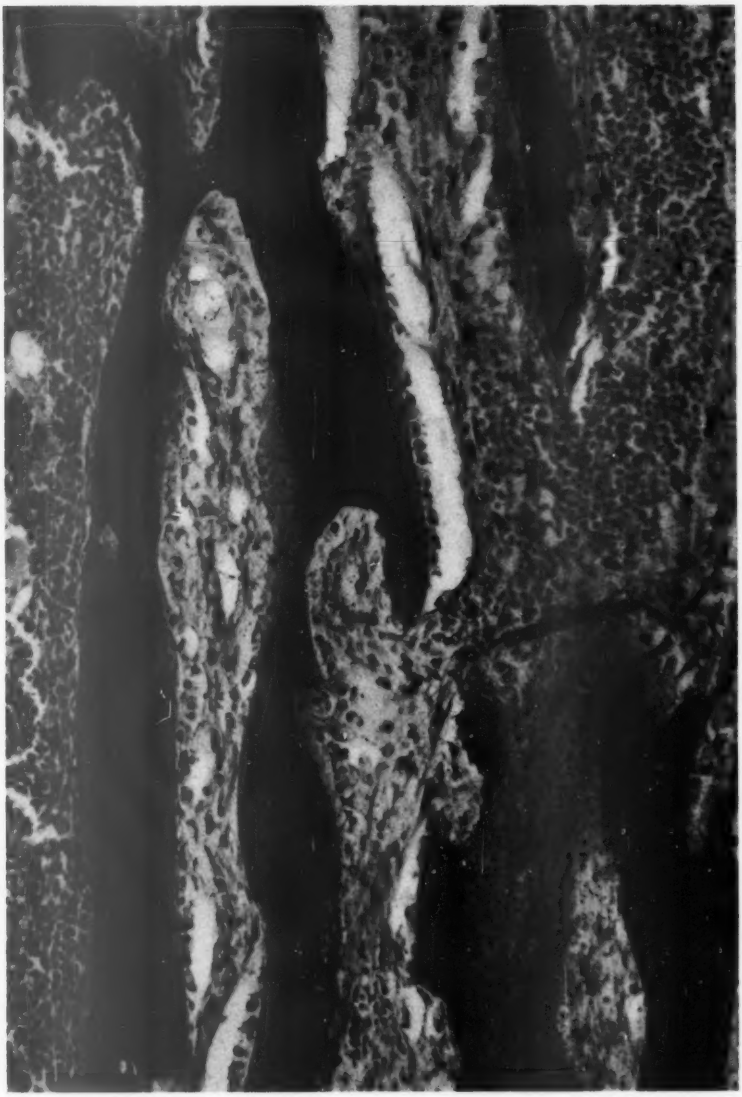


2

Carttar, McLean, and Urist Dietary Calcium and Phosphorus

PLATE 52

FIG. 3. The spongiosa of the animal reared on Sherman's diet considered adequate with respect to calcium, from the area enclosed in the rectangle in Figure 2. Of note are the wide osteoid borders of the new bone trabeculae. × 350.

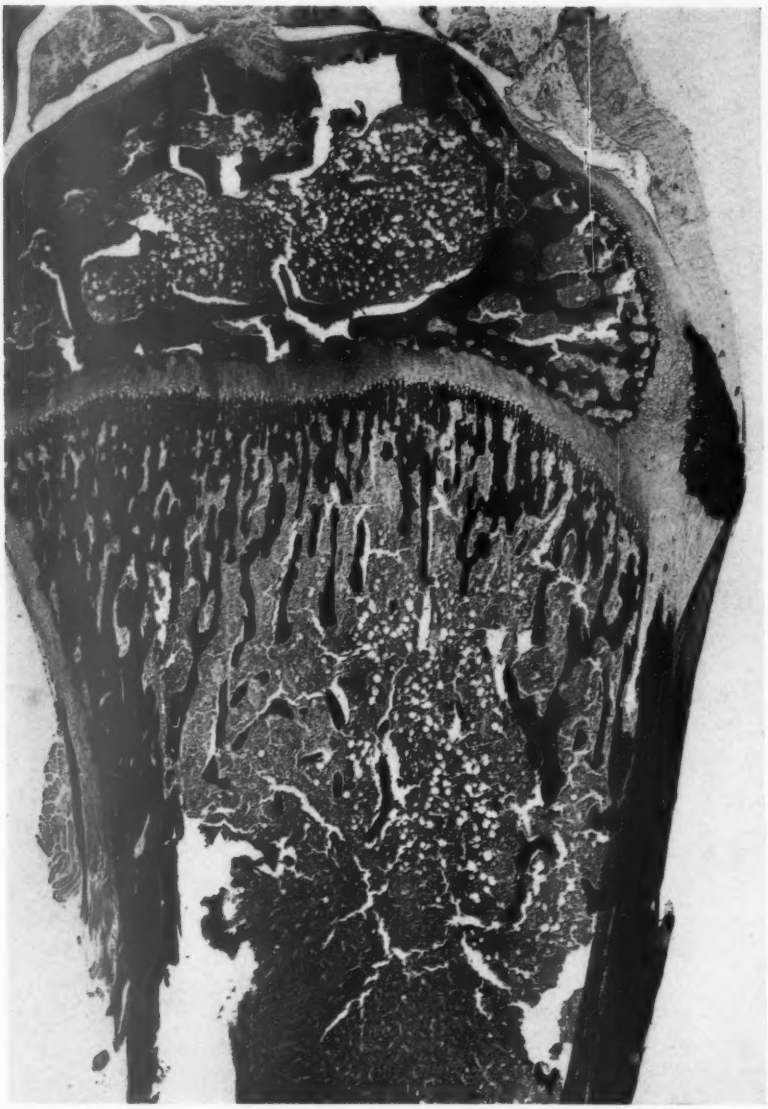


3

Carttar, McLean, and Urist Dietary Calcium and Phosphorus

PLATE 53

FIG. 4. Photomicrograph of the upper end of the tibia of a littermate of the rat shown in Figure 1, reared on a diet containing the optimum amount of calcium. As compared with the tibia shown in Figure 2, the zone of provisional calcification of cartilage is several cells deeper, the metaphysis is longer, the bone trabeculae are heavier and more numerous. Undecalcified section stained in silver-hematoxylin and eosin. × 25.

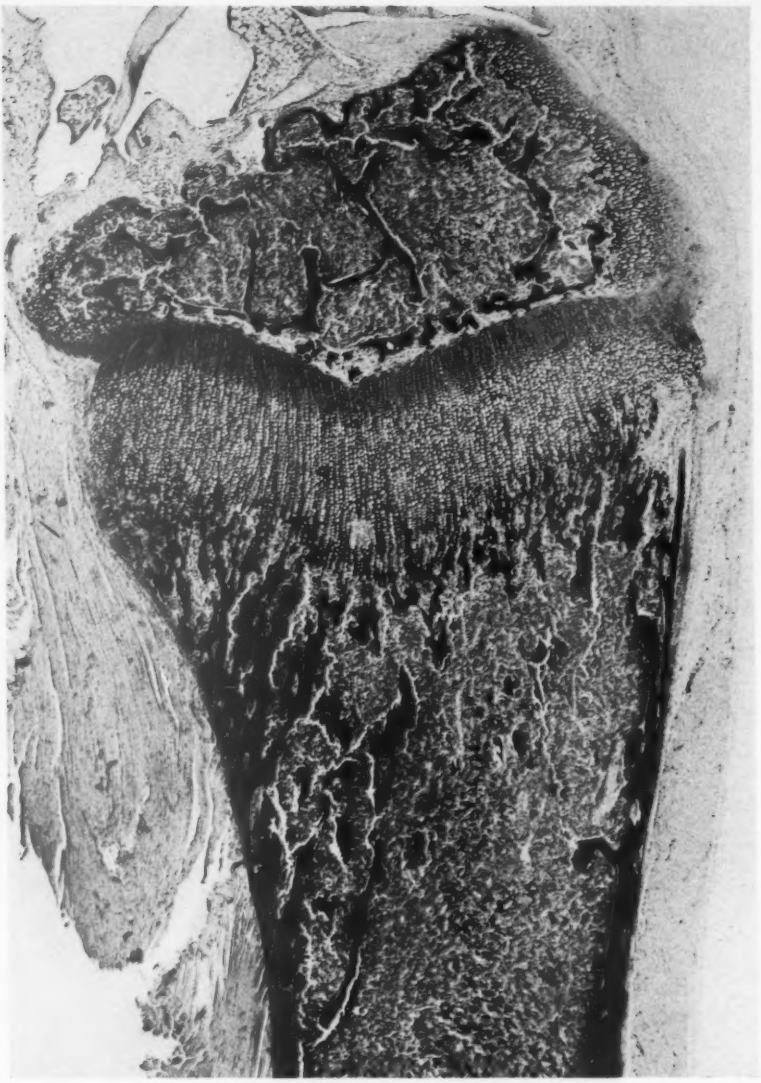


4

Carttar, McLean, and Urist Dietary Calcium and Phosphorus

PLATE 54

FIG. 5. The upper end of the tibia of a rat reared on a "normal" diet supplemented with 8 per cent $CaCO_3$, during the period between 3 and 6 weeks of age. Of note are the extreme thickness of the epiphyseal cartilage, the failure of provisional calcification of the hypertrophic cartilage cells, and the absence of the normal process of cartilage removal and endochondral ossification. This picture may be described as "high calcium rickets." Undecalcified section stained in silver-hematoxylin and eosin. × 25.

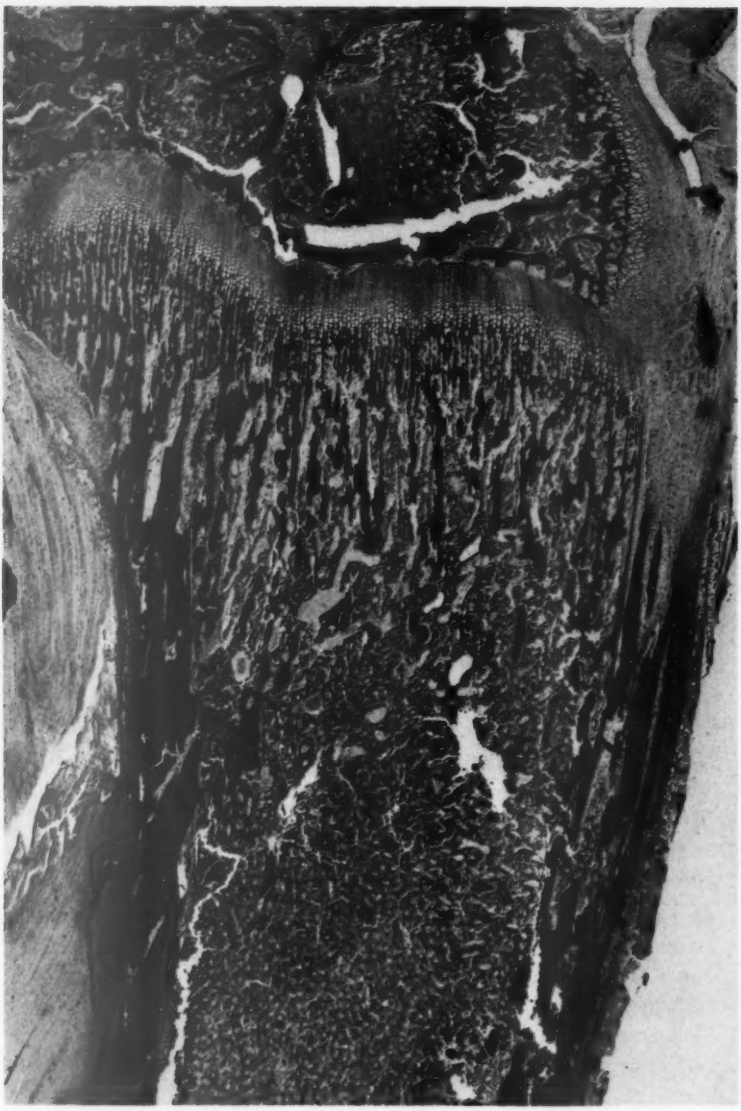


5

Carttar, McLean, and Urist Dietary Calcium and Phosphorus

PLATE 55

FIG. 6. Photomicrograph of the upper end of the tibia of a rat of the same age as the one shown in Figure 4, but reared on a similar diet fortified with approximately 0.6 per cent more phosphorus than is present in Sherman's adequate or optimum diets. The additional phosphorus protected the animal against rickets.



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Carttar, McLean, and Urist Dietary Calcium and Phosphorus

CARCINOMA OF THE PANCREAS
A Clinical and Pathologic Analysis of Thirty-nine Autopsied Cases*

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Despite the many recent advances in the clinical diagnosis and treatment of cancer, carcinoma of the pancreas remains one of the gravest of medical and surgical problems. The clinical picture, upon which early diagnosis and successful treatment depend, is often confused by the inconsistency and obscurity of the symptoms referable to the pancreas, and rendered misleading by symptoms resulting from the presence of metastases in various parts of the body. In addition, the apparently late onset of symptoms of sufficient severity to induce the patient to call upon the services of a physician further adds to the difficulty.

Carcinoma of the head of the pancreas has received considerable attention for many years, primarily because of the characteristic involvement of extra-hepatic bile ducts with the production of jaundice. Consequently, it is better understood and more amenable to surgical treatment than cancer at other sites in the pancreas. Carcinoma of the body and tail of the pancreas, on the other hand, has only recently come to the fore by a series of papers by Ransom,[1] Duff,[2] Levy and Lichtman,[3] and Russum and Carp,[4] who have tended to split carcinoma of the body or tail of the pancreas from carcinoma of the head, and to consider it, rightly or wrongly, as a separate entity. The silent nature of the primary tumor, and the frequency of symptoms referable to metastases, have been emphasized especially by Duff.[2] Other authors have emphasized various symptoms and physical findings often seen in association with cancer of the body or tail of the pancreas. It is with these assertions in mind that the analysis of the present series of cases is undertaken, the primary object being to determine if these cases are in line with series reported earlier. The clinical and pathologic differences between carcinoma of the head of the pancreas and that of the body or tail are emphasized when necessary.

Carcinoma of the pancreas forms between 1 and 2 per cent of all cancers,[5-7] and between 0.3 and 0.75 per cent of all cases autopsied. The present series is composed of 39 consecutive cases of carcinoma of the pancreas among a total of 828 cases of malignant tumors con-

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tained in the autopsy records of the Pathological Institute, McGill University, from the year 1939 to 1947 inclusive. It follows consecutively on the series of cases of carcinoma of the pancreas reported from this Institute by Grauer[8] in 1939. Among the cases in the present series were 32 males and 7 females, a ratio of about 4.5:1. The average age for the entire series was 60 years, with the largest number of cases occurring during the 6th and 7th decades, 25 in all. The remaining cases were scattered between the 4th and 9th decades with 2 cases in the 4th decade, 5 in the 5th, 5 in the 8th, and 1 in the 9th. The youngest patient was 34, and the oldest, 82 years of age. The age distribution corresponds well with that of other reported series.

Pathologic Features

The head of the pancreas alone was involved by the primary tumor in 21 of the 39 cases, the body in 9, and the tail in 2. The remaining 7 cases were those in which the exact site of origin of the tumor in the pancreas could not be determined accurately because of the massive local extension of the cancer and involvement of the local lymph nodes. In 3 cases there was involvement of the head and body, the body and tail in 2 others, and in 2 cases, the whole pancreas appeared to be involved. There are, therefore, in all, 21 cases in which the head of the pancreas alone was the site of the primary tumor, compared to 13 cases in which the body or tail of the pancreas, or both, were involved. In 5 other cases the head was involved in addition to other parts of the pancreas. The ratio of the two former groups of cases, 3:2, is at variance with the usual reported ratio of 3:1, head of pancreas to body or tail,[1,3] although it corresponds more favorably with the cases of Duff,[2] who also offers a logical explanation for the variation.

Macroscopic Appearance. In the majority of cases, the tumor formed a hard nodular mass in the substance of the pancreas, in some instances entirely within the pancreas and surrounded by a narrow rim of distorted pancreatic tissue. Generally, however, the tumor had spread beyond the pancreas and had become continuous with adjacent enlarged cancerous lymph nodes, and sometimes with masses of tumor lying in the posterior wall of the abdomen. The average size of the primary, exclusive as far as possible of the adjacent spread, was 6.1 cm. in diameter, with a variation between 3 and 17 cm. As with Grauer's series,[8] no relationship between the site and size of the tumor was observed, although D'Aunoy, Ogden, and Halpert[9] reported larger tumors in the head of the pancreas than in the body or tail. The con-

sistency, as has been indicated, was usually hard or stony hard. In only 4 cases was the tumor described as being of a soft, friable type, and these were subsequently shown to be highly cellular with little fibrous stroma. Because of the usually scirrhous nature of the tumor, areas of degeneration and necrosis are seen only infrequently. In the present series, only 8 cases showed degenerative changes, taking the form of small areas of cystic and gelatinous change in all of the hard tumors, and necrosis and hemorrhage in the soft tumors. One case showing most extensive necrosis was that of a cystadenocarcinoma of the tail of the pancreas, where the tumor was converted into a large hemorrhagic cyst with a shaggy lining of necrotic tumor tissue.

Microscopic Appearance. Carcinoma of the pancreas may assume a variety of histologic types dependent upon the structure from which it arises. The commonest form is adenocarcinoma, arising from pancreatic duct epithelium and represented in the present series by 28 cases. These tumors are characterized by the formation of duct-like spaces lined by columnar or cuboidal cells, in one or more layers. The stroma separating these formations is usually densely collagenous. The degree of differentiation may vary considerably from tumors in which the ducts are well formed and lined by a single row of regular columnar cells to those in which ducts are formed poorly, if at all, and in their place are cords and groups of tumor cells showing marked irregularity with many abnormal forms and giant cells. In some instances of the present series, the histologic picture differed considerably in different parts of the same tumor. In one area, well differentiated duct formations were seen, separated by a dense fibrous stroma; in other areas there was an abrupt change to a more anaplastic growth, with the epithelial cells arranged in sheets between which were broad bands of dense connective tissue. This variable picture was seen in 10 of the cases of duct carcinoma in this series. Of the remaining 18 cases, 7 were considered as being well differentiated and 11 as undifferentiated. No attempt was made otherwise to grade the tumors histologically.

The second histologic type of carcinoma of the pancreas is that in which the neoplasm resembles microscopically the parenchymatous structure of the pancreas, the acinar cell carcinoma. It is represented in this series by but 6 cases, and, therefore, is seen much less often than duct carcinoma. These tumors are composed of small rounded or spindle-shaped cells arranged in small groups separated by a fine fibrous stroma. This arrangement often bears a striking resemblance to the normal acinar structure of the pancreas. In several of the

present cases a transition between normal pancreatic acini and neoplastic tissue was observed. Cell variation was marked in some cases, with numerous abnormal forms, tumor giant cells, and many mitotic figures. As a rule, the stroma was scanty and composed of fine fibrous connective tissue. Degenerative changes, in the form of necrosis and hemorrhage, sometimes were seen.

Carcinoma arising from the islet structures of the pancreas is the rarest form of carcinoma of the pancreas. This histologic type was first reported by Wilder and his associates in 1927.[10] Since that time many case reports have appeared in the literature, and almost all series reported include one or more cases of this histologic type. In this series, there was only one case with the histologic picture of an islet cell carcinoma. The microscopic features were similar to those described by other authors, namely, sheets of small polyhedral or columnar cells possessing oval, darkly staining nuclei. The arrangement of the cells was mainly haphazard, but in some areas showed a tendency to form short rows or small rosettes, separated by a fine collagenous stroma. It is unfortunate that the clinical data bearing upon this case are meager, and that a possible disturbance of carbohydrate metabolism was not determined. The diagnosis in this case was not made until microscopic examination following autopsy.

As was mentioned earlier, in 3 cases of this series the histologic picture was that of an undifferentiated neoplasm, and examination of the available sections failed to reveal any features which would place them in any of the above groups without doubt. Microscopically, all 3 cases showed undifferentiated tumor cells varying greatly in size, shape, and appearance, with numerous giant cells and mitotic figures. Bizarre hyperchromatic nuclei were common. In 2 cases the stroma was moderately fibrous, separating irregular groups and columns of epithelial cells, suggestive of duct origin, although careful search revealed no evidence of duct formation in either the primary tumor or the metastases. In the third case, the stroma was scanty, and the cells were arranged in large sheets, suggestive of acinar cell carcinoma.

One other case in this series showed microscopically numerous small cyst-like spaces, variable in size and shape and lined by a single row of tall-columnar epithelium, with deeply staining, basilar placed nuclei and pale-staining cytoplasm. This orderly picture was replaced in many areas by solid masses of pleomorphic tumor cells showing atypical giant cells and mitotic figures. The whole central portion of the tumor was completely necrotic. Metastatic lesions from this tumor were more typical of duct carcinoma, indicating the probable origin of the tumor. The history of this case dated back 5 years from the time of death,

and earlier biopsies revealed that this tumor was originally a benign cystadenoma of the pancreas which subsequently underwent malignant change.

Local and Metastatic Extension

Much has been published regarding the spread of carcinoma of the pancreas, and the differences which are purported to exist in this respect between carcinoma of the head of the pancreas and carcinoma of the body and tail. It is with these differences in mind that the local and metastatic spread of the present series of cases has been analysed.

Local Extension (Table I). Observations on this series of cases indicate that the direction and effect of local extension in carcinoma

TABLE I

Local Extension of Carcinoma of the Pancreas
(Total of 39 Cases)

Structures involved by local spread	Head of pancreas (21 cases)	Body and/or tail (13 cases)	Head and other (5 cases)	Total cases
Bile duct	16	2	2	20
Duodenum	13	3	1	17
Peritoneum	8	9	5	22
Splenic vein	3	6	0	9
Portal vein	3	3	1	7
Posterior abdominal wall	3	4	2	9

of the pancreas are dependent upon the location of the tumor in the pancreas, and its relationship to adjacent structures. In cancer of the head of the pancreas, the common bile duct is involved frequently because of its intimate relationship. Invasion of the common bile duct occurred in 16 of the 21 cases of carcinoma of the head of the pancreas, and in only 2 cases in which the tumor involved the body or tail alone. Invasion into the wall of the duodenum was seen similarly in 13 cases of the former, and in only 3 of the latter. On the other hand, involvement of the splenic vein occurred in 6 cases of carcinoma of the body or tail of the pancreas, and in only 3 of carcinoma of the head.

Invasion of the peritoneum, with subsequent spread throughout the peritoneal cavity, occurred in 8 cases of cancer of the head, in 9 of the 13 cases in which the body or tail was involved, and in all 5 cases in which the head of the pancreas was involved as well as other parts of the pancreas. Spread to, and invasion along, the posterior wall of the abdomen was seen more frequently in the latter group, with 4 of 13 cases as compared to 3 of 21 cases of carcinoma of the head of the pancreas. Portal vein invasion was observed in 3 cases of carcinoma of the head of the pancreas and in 3 cases in which body or tail of the pancreas was the site of the primary tumor.

Absence of extension beyond the pancreas was observed in 3 cases

of the present series. All 3 patients underwent operation for partial or total pancreatectomy shortly before death.

Metastatic Spread (Table II). The commonest site of metastatic lesions in carcinoma of the pancreas, irrespective of the location of the

TABLE II

Metastatic Spread of Carcinoma of the Pancreas
(Total of 39 Cases)

Location of metastases	Head of pancreas (21 cases)	Body and/or tail (13 cases)	Head and other (5 cases)	Total cases
Local nodes	15	13	5	33
Mediastinal nodes	1	6	1	8
Cervical nodes	0	2	1	3
Liver	11	11	1	23
Spleen	0	2	1	3
Gallbladder	0	1	1	2
Diaphragm	1	7	0	8
Lungs	4	8	1	13
Pleura	1	4	1	6
Pericardium	1	1	1	3
Heart	0	1	1	2
Thyroid	1	1	0	2
Salivary gland	0	2	0	2
Skin	2	2	0	4
Bones	2	5	0	7
Adrenal gland	2	3	1	6
Kidney	2	4	2	8
Muscles	0	1	0	1
Dura	0	1	0	1

primary, was the local abdominal lymph nodes, which include the peripancreatic, para-aortic, and mesenteric groups. Metastasis to these nodes was seen in 15 of the 21 cases of carcinoma of the head of the pancreas and in all cases of carcinoma at other sites (18 cases). The next most common site of metastasis was the liver, occurring in 11 cases of the former group and 12 of the latter. Metastasis to more distant structures occurred considerably less often than to the above-mentioned sites, but the differences between cancer of the head of the pancreas and cancer at other sites in the pancreas with respect to both location and frequency were more apparent. Lymphatic dissemination generally followed the course of the thoracic duct from the celiac and para-aortic nodes. The mediastinal lymph nodes were secondarily invaded by carcinoma in a total of 8 cases, of which only one was from a carcinoma of the head of the pancreas. Further extension to the supraclavicular and cervical lymph nodes on the left side occurred in 3 of the cases. All were from carcinomas in the body of the pancreas.

Secondary invasion of the lungs was seen in 13 cases, 4 of which were from primaries in the head of the pancreas. Metastasis to the lungs may occur in two ways: by extension of tumor into the lung

from lesions already present in the mediastinal lymph nodes; and by the blood stream, with lodgment of tumor emboli in the parenchyma of the lung. Extension from the mediastinal nodes usually gives rise to large masses of tumor in the hilus of the lungs, whereas hematogenous spread results in multiple nodules at the periphery of the lung. In the present series, peripheral lung metastases were seen in 6 cases in the absence of mediastinal lymph node involvement; in 7 cases masses of secondary tumor were located in the hilus of the lung, in association with large cancerous lymph nodes. This appearance confused the clinical picture in 3 instances, an erroneous diagnosis of bronchogenic carcinoma being arrived at, supported in one case by a positive bronchoscopic biopsy. Pathologic examination of all 3 cases revealed large secondary tumors in the hilus of the lungs with occlusion of one or more of the main bronchi.

Metastatic lesions of the pleura were seen in 5 cases of cancer of the body or tail of the pancreas and in only one of carcinoma of the head alone. Diaphragmatic secondaries occurred in a total of 8 cases, only one being from a carcinoma of the head of the pancreas. The association of pleural and diaphragmatic secondaries is probably close, and there seems little doubt that in some cases pleural metastases result from spread of the tumor through the diaphragm as well as direct spread from adjacent metastases in the lungs. Other metastases occurred to the skin, bones, adrenal glands, and kidneys in 2 cases of carcinoma of the head of the pancreas, and in 2, 5, 4, and 6 cases, respectively, in which other parts of the pancreas were involved. Involvement of the pericardium occurred by direct spread from mediastinal lymph nodes in 3 cases. The thyroid gland showed secondary carcinoma in 2 cases, in one of which the head of the pancreas was the site of the primary. Other sites of secondary tumor from cancers of the body or tail of the pancreas were spleen in 3 cases; gallbladder, heart, and salivary glands in 2 cases each; skeletal muscles, and dura in one.

The widespread nature of these more distant metastases and their more frequent occurrence from a carcinoma primary in the body or tail of the pancreas supports the earlier contentions of Ransom,[1] Duff,[2] and others. These metastases frequently give rise to signs and symptoms which confuse the clinical picture and may lead to a wrong diagnosis. Histologically, cancer of the body or tail of the pancreas does not differ from that of the head of the pancreas. The reason for the more widespread extension observed at autopsy possibly lies, therefore, in the longer duration of growth permitted by its location in the pan-

creas away from any vital structure. On the other hand, Duff[2] has advanced explanations based on the anatomical location and relations of carcinoma arising in the body and tail of the pancreas and the ready access of such tumors to pathways of spread to near and distant sites.

Associated Pathologic Findings

Changes in other structures concurrent with pancreatic carcinoma are those which result from the presence of the tumor or its metastases, and their effect upon the structures with which they come into contact. Findings in the pancreas uninvolved directly by the cancer, apart from local distortion due to an expanding tumor, depend largely upon the location of the tumor in the pancreas. In cancer of the body or tail of the pancreas, the head appears relatively unaffected. Carcinoma of the head of the pancreas, however, usually results in early occlusion of the pancreatic duct, following which atrophic changes occur in the body and tail of the pancreas. These changes take the form of atrophy and replacement fibrosis of the acinar tissues, and dilatation of the pancreatic ducts. These histologic changes were observed in 13 cases of the present series, of which 11 were carcinoma of the head of the pancreas and 2 of carcinoma of the body.

In addition to metastatic lesions in the liver, a variety of other changes were observed, many of which were related to obstruction of the biliary system. Fatty degeneration was seen in 9 cases, cholangitis in 5, focal necrosis of the liver cells in 3 cases. Simple atrophy of the liver cells was described in 3 other cases.

Invasion of the peritoneum by tumor led to ascites in 11 cases, 6 of which were in cases of carcinoma of the head of the pancreas. Bile peritonitis was seen in 4 cases of carcinoma of the head of the pancreas. Infective purulent peritonitis was present in 9 cases of this series and hematoperitoneum in 3 others. In all of these cases, either laparotomy alone or an attempt at surgical removal of the pancreas preceded death by a few hours or days.

Duodenal ulceration, resulting from direct invasion of the wall of the duodenum by the cancer, was seen in 8 cases. In 6 the tumor was located in the head of the pancreas, and in 2 cases was in the body of the pancreas and had invaded the wall of the third part of the duodenum. In one of these cases, the tumor had eroded the wall of the duodenum and had produced a fungating growth almost completely occluding the lumen.

The frequent occurrence of venous thrombosis in cases of carcinoma of the pancreas has been a subject of much discussion in recent years.

Sproul,[11] Levy and Lichtman,[3] and Kenney[12] have laid particular stress on this feature of the disease. In Sproul's series, venous thrombosis was observed in 9 of his 16 cases of carcinoma of the body or tail of the pancreas, as compared to an incidence of between 15 and 25 per cent in other abdominal cancers. The conclusion of these authors was that there may be some interference with the mechanism of blood clotting associated with these tumors, although, at present, there is no substantial proof for such an assumption.[13] In this series, venous thrombosis was observed in a total of 13 cases. In 5 of these, thrombosis occurred in the portal and splenic veins, and appeared to be due to invasion of the venous wall by cancer. Two others may be considered as coincidental findings, one being a case of coronary thrombosis and the other of mural thrombosis in the left ventricle of the heart, associated with marked myocardial changes. The 6 remaining cases of venous thrombosis include thrombosis of the iliac veins, mesenteric veins, and, in one case, the prostatic venous plexus. Of these cases, the head of the pancreas was the site of the primary in 3, the body in one, and the whole pancreas was involved in 2.

Clinical Features

Analysis of Symptoms

Weakness and Loss of Weight. The commonest initial complaint in the present series of cases was loss of weight, frequently accompanied by weakness. Loss of weight was observed in 30 of the 39 cases and was found in association with rapidly progressive weakness in 13. The average loss of weight in 21 of the cases was 26.4 lbs., with an average duration of 6 months, based on all cases. In 22 of the 30 cases, the weight loss was of 6 months' duration or less. No specific differences in this respect were noted between carcinoma of the head and cancer at other sites in the pancreas. It is thought that the symptoms loss of weight and weakness are those of malignancy in general, although Ingelfinger[13] has stressed their importance in carcinoma of the pancreas.

Abdominal Pain. Abdominal pain occurred in 28 cases, and in this series appeared to be of much greater symptomatic importance than loss of weight. Indefinite abdominal pain formed the largest group, with 12 of the 28 cases. In these cases, the pain exhibited no specific localization, and was of a vague character, often with intermittent, crampy episodes. In the remaining 16 cases, localization was more definite, epigastric in 7, right upper quadrant in 6, left upper quadrant

in 2, and the left lower quadrant in 1 case. The pain was most frequently described as a vague ache, but was specified as being crampy in 3 cases, intermittent in 4, and stabbing in 1. Some relationship to meals was seen in 10 cases, the pain coming on at an indefinite time after meals. In 11 cases, radiation of the pain to the scapulae and lumbar regions of the back was observed. Radiation of the pain to the left occurred in 4 cases.

Certain natural relationships of the pain to the site of the carcinoma were noted. In 10 of the 13 cases exhibiting epigastric or right upper quadrant pain, the cancer was located in the head of the pancreas. On the other hand, radiation of the pain to the back was seen in 7 cases of carcinoma of the body or tail of the pancreas, compared to 4 cases in which the head of the pancreas was involved. Radiation of the pain to the left occurred in 2 cases of cancer of the head of the pancreas, and in 2 cases of cancer of the body of the pancreas. The average duration of the pain prior to death was 7 months, with a variation between 3 days and 2 years. In cases of longer duration the pain was usually intermittent.

Abdominal pain, rapid weight loss, and weakness appear to be the presenting symptoms in many cases of carcinoma of the pancreas, regardless of the site of the tumor.[1-4,14,15] Radiation of the pain to the back has been emphasized by several authors[16,17] as being an important symptom in carcinoma of the body of the pancreas, although in this series it was not prominent. On the other hand, it is worth pointing out that the occurrence of abdominal or back pain in a total of 15 of the 21 cases of carcinoma of the head of the pancreas denies the oft-repeated statement that cancer arising in this location is characterized by "painless jaundice." The cause of abdominal pain in carcinoma of the pancreas is generally considered to lie in the invasion of the celiac nerves by tumor.[18]

Symptoms Referable to Disturbance of Bile Excretion. Among the initial complaints, jaundice was encountered in a total of 15 cases, clay-colored stools in 12 cases, and dark-colored urine in 8 others. The average duration of jaundice was found to be 3.9 weeks, exclusive of 2 cases in which jaundice was intermittent over periods of 6 months and 2 years. The average duration of dark-colored urine was 5 weeks and of clay-colored stools, 7 weeks. All cases were those of carcinoma of the head of the pancreas with obstruction of the common bile duct being the usual cause. In 3 cases, however, the common bile duct was patent at autopsy, and the cause of the obstruction was not determined, although external pressure by the primary tumor appeared probable. This triad of symptoms is obviously that of cancer of the head of the

pancreas rather than of the body or tail. Pain is a usual accompanying symptom, despite the old impression of the importance of painless jaundice. Berk[7] has estimated the incidence of painless jaundice in carcinoma of the head of the pancreas as being about 20 per cent. In this series, there were only 3 instances of painless jaundice, in one of which the jaundice had occurred intermittently over a period of 23 months.

Gastro-intestinal Symptoms. Constipation was seen more commonly than diarrhea, with 12 instances of the former to 5 of the latter. Alternating constipation and diarrhea were observed in one case. Four of the cases presenting constipation were those in which the tumor was located in the head of the pancreas, and in 3 of the 5 cases of diarrhea also the neoplasm was located in the head.

Nausea and vomiting, usually of an intermittent type, were encountered in 13 of the 39 cases of the series, with an average duration of 5 months. In 9 cases the cancer involved the head of the pancreas, and of the remaining 4, the body of the pancreas was the primary site in 2, and 2 others were cases of multiple involvement. In only one case was there recorded a single instance of hematemesis, which occurred shortly before admission in a carcinoma of the head of the pancreas. In Duff's series,[2] hematemesis was noted in 8 of his 16 cases of cancer of the body or tail of the pancreas, and in one case of carcinoma of the head of the pancreas. Vomiting is reported only infrequently, and hematemesis not at all in other series of cases.[1,3]

Symptoms Referable to Chest Disease. Cough, usually associated with the production of variable amounts of sputum, and in 2 instances with hemoptysis, was seen infrequently in the present series. This symptom was observed in 4 cases and 3 of these were subsequently diagnosed as bronchogenic carcinoma. These cases have been dealt with under an earlier heading. In all, the pulmonary lesions were shown by roentgenologic examination of the chest.

Analysis of Physical Findings

Palpable Superficial Nodules. Palpable superficial axillary, supraclavicular, cervical, or inguinal lymph nodes were present in 9 cases, of which only 2 were cases in which the primary tumor was confined to the head of the pancreas. Pathologic confirmation of the presence of cancer in the enlarged lymph nodes was provided in only 5 of these cases. Multiple metastatic skin nodules were seen in 4 cases; 2 in which the primary tumor was in the head of the pancreas and 2 in the body or tail.

Palpable Mass. A palpable abdominal mass was present on admis-

sion in 21 of the 39 cases of this series. The average size recorded in 8 cases was 5 cm. with a range of from 2.5 to 10 cm. The sites were the right upper quadrant in 10 cases, epigastrium in 8, left upper quadrant in 2, and in one other case the exact site was not stated. Carcinoma of the head of the pancreas accounted for many of the cases with a mass in the upper part of the abdomen, for 7 of the 10 right upper quadrant masses, and for 6 of the 8 cases of epigastric masses. Cancers of the body and tail of the pancreas were responsible for the masses in the left upper quadrant.

Palpable Enlargement of the Liver. Definite enlargement of the liver was present in a total of 23 cases, with an average enlargement of 4 cm. below the right costal margin. Gross metastases to the liver were found at autopsy in only 11 of these cases. Findings in the remaining 12 cases varied, 6 showing marked cholangiectasis due to common bile duct occlusion. In 4 other cases, the common bile duct was apparently patent but evidences of moderate bile stasis were seen on microscopic examination. Marked cholangitis was observed in one case and in another marked fatty degeneration. It is interesting to recall that metastasis to the liver occurred in a total of 23 cases, and that in 12 of these, the liver was not clinically enlarged.

Analysis of the incidence of gallbladder enlargement was unsatisfactory in this series. It is possible that in some of the cases of hepatic enlargement in the absence of metastases, an enlarged and tense gallbladder was mistaken for the liver edge. It is now generally agreed that clinical enlargement of the gallbladder is present in about 50 per cent of the cases of carcinoma of the pancreas with jaundice.

Palpable Enlargement of the Spleen. Clinical enlargement of the spleen was seen in only 2 cases of the present series, both of which showed at autopsy areas of infarction due to thrombosis caused by invasion of the splenic vein by tumor. The primary arose in the body of the pancreas in one case, and in the head in the other. At autopsy, 2 other cases of splenic infarction were revealed which did not give rise to enlargement of the spleen.

Ascites. Examination on admission to the hospital showed ascites to be present in 8 cases, with one doubtful case in addition. This figure can scarcely be compared with those discovered at autopsy, because in many instances the results of operative procedures led to hematoperitoneum, and infective and bile peritonitis. The head of the pancreas was the site of the primary tumor in 2 cases, the body or tail in 5, and in one other case the tumor involved the head and body. The single doubtful case was that of a cancer of the head of the pancreas.

Roentgenologic Findings. Serial examination with barium was performed in 24 cases, 11 being reported as negative. In 8 other cases, distortion of the duodenal loop and normal outline of the stomach were reported, due in most instances to pressure upon these parts by an extrinsic tumor mass in the region of the pancreas. In one case a definite shadow was seen within the loop of the duodenum. In 2 other cases, gastric retention due to pyloric obstruction was seen. Other findings included ulceration of the duodenum in one case, and in another a fungating tumor mass in the third part of the duodenum. As would be expected, the majority of cases with positive findings were carcinomas of the head of the pancreas.

Barium enema examination was undertaken in 10 cases and gave positive information in only one. This case showed evidence of extrinsic pressure upon the transverse colon, subsequently shown to be due to a massive cancer of the head of the pancreas.

Roentgenologic examination of the chest was positive in 7 of 18 cases, revealing densities in various parts of the lung field. In one case pleural effusion was the main finding. As was indicated earlier, positive chest findings in 3 cases aided in the misdiagnosis of bronchogenic carcinoma.

The incidence of positive roentgenologic findings in pancreatic carcinoma is stated to be approximately 50 per cent.[7,19] Successful results are more or less confined to cancer of the head of the pancreas.

The Final Clinical Diagnosis

Of the 39 cases studied in this report, 4 were undiagnosed at time of death. One of these seems particularly interesting, being the case of a 72-year-old man who was admitted to the hospital with a fracture of the left tibia and who died a few weeks later of bronchopneumonia. At no time were there symptoms referable to the pancreas. Autopsy revealed a primary carcinoma of the body of the pancreas, measuring 5.5 by 3.5 by 4 cm., from which metastasis to the liver and regional lymph nodes had occurred. In 18 other cases, carcinoma of the pancreas was diagnosed before death, the site of the tumor being specified as the head in 7. Diagnosis on purely clinical grounds alone was made in only 6 of the cases; in the remaining 12, the diagnosis was established by exploratory laparotomy.

In 17 cases of the series the clinical diagnosis was erroneous. In 5 the clinical diagnosis was carcinomatosis of undetermined origin. Four others were positively diagnosed as carcinoma of the stomach, 2 as acute pancreatitis, and 1 case each as carcinoma of the common bile duct, carcinoma of the gallbladder, and Hodgkin's disease. Of

particular interest are the 3 cases diagnosed as bronchogenic carcinoma.

With reference to the site of the carcinoma in the pancreas, analysis reveals that of the 21 cases of carcinoma of the head of the pancreas, 12 were clinically diagnosed correctly, and 5 of these preoperatively. On the other hand, only a single case was diagnosed preoperatively for cancer at other sites in the pancreas. Five others were correctly diagnosed postoperatively.

Summary

A detailed analysis of 39 cases of carcinoma of the pancreas has been made with the object of determining whether this series is in agreement with other series reported. Included were 21 cases in which the head of the pancreas alone was occupied by the carcinoma, 13 cases in which the tumor involved the body and/or tail of the pancreas, and 5 cases in which the tumor involved the head and body or the whole pancreas. The first two groups were compared.

The gross and microscopic pathology of carcinoma of the pancreas is well known and is in no way altered by the present series of cases. No differences were observed in the histologic appearances of carcinoma of the head of the pancreas as compared with carcinoma of the body or tail of the pancreas.

Carcinoma of the head of the pancreas differs materially from cancer at other sites in the pancreas, both in the local effects and in extent of the metastatic lesions. Differences in the local effects depend upon the location of the cancer and its relations to adjacent structures. Carcinoma of the body or tail of the pancreas metastasizes more often and more widely than carcinoma of the head of the pancreas. This feature frequently gives rise to misleading symptoms which obscure a correct diagnosis.

Special emphasis is laid upon 3 cases wrongly diagnosed as bronchogenic carcinoma, one of which was supported by a positive bronchoscopic biopsy. In one other case, a carcinoma of the body of the pancreas was entirely unsuspected until autopsy was performed.

Venous thrombosis was not a prominent feature in the present series of cases. It was observed in about the same frequency as would be expected with other abdominal cancers.

The variability of the clinical features of the cases in this series re-emphasizes the great difficulty of early diagnosis. This difficulty appears due primarily to the inconstancy of the symptoms and signs, and also to the relatively late onset of symptoms of sufficient severity to bring the patient to a physician. This is particularly in evidence in carcinoma of the body and tail of the pancreas.

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